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THE BIOAVAILABILITY OF IRON SOURCES AND THEIR UTILIZATION IN FOOD ENRICHMENT

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by

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FOREWORD

The Life Sciences Research Office (LSRO), Federation of American Societies for Experimental Biology (FASEB), provides scientific assessments of topics in the biomedical sciences. These reports are based upon comprehensive literature reviews and the perceptive observations of knowledgeable scientists engaged in work in the field. Although LSRO reports are recognized by FASEB as contributions to societal needs and most LSRO consultants are members of FASEB constituent societies, the reports do not necessarily reflect the views of the members of its six constituent societies. However, the report has been reviewed for policy matters by the LSRO Advisory Committee which includes representatives of each society.

This technical report was prepared for the Division of Nutrition, Bureau of Foods, Food and Drug Administration by the Life Sciences Research Office, FASEB, in accordance with the provisions of FDA contract number 71-294. As an earlier part of this contractual effort LSRO conducted a review of current scientific knowledge concerning dietary iron in iron overload syndromes and related iron storage phenomena in man and experimental animals (Waddell *et al.*, 1972a). That study concluded that the proposed increase in the iron content of enriched flour and flour-containing dietary items (Federal Register, 1971) will have little or no effect on the accumulation of iron by normal males, and that the extra iron *per se* will not initiate the development of hemochromatosis or other hereditary iron storage disorders, although it may accelerate the course of these diseases. The report noted that additional research would be required to resolve conclusively the effect of the proposed level of dietary iron on iron accumulation. In another report, two protocols of clinical research were designed to elucidate the possible hazards of increasing the level of iron enrichment of cereal products (Waddell *et al.*, 1972b).

In the conduct of these LSRO review studies, it became evident that the form of iron ingested and the composition of the diet markedly influence the amount of iron absorbed and the effectiveness of increasing the level of dietary iron in preventing or reducing the prevalence of iron-deficiency in the population was questioned. For these reasons the entire subject of the bioavailability of iron sources used in the enrichment of foods has been reviewed.

The present report is a comprehensive analysis of the subject of the bioavailability of iron sources and should be useful in establishing a scientific basis for future decisions by FDA as to the forms of iron most suitable for iron enrichment of various types of foods.

The report was written by Dr. James Waddell who served as a special consultant to the staff of LSRO for this study. We acknowledge the advice provided by the consultants names in Section VIII; their suggestions assisted in preparing this report.

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SUMMARY

The principal objective of this report has been to review the program of iron enrichment of foods and to assess the suitability of the different iron compounds which have been used. Suitability has been judged from the standpoint of the bioavailability of the form of iron used and the effects of the iron compounds on the foods to which they were added. It has been found that, essentially, the complete story of iron enrichment is comprised in the use of four compounds added to foods derived from the cereal grains.

The first four sections of the report are a review of the development of knowledge of iron metabolism over the last half century; the methods by which iron absorption has been measured; and the factors which influence the absorption of iron from the digestive tract. This background information, from human and animal research, is of value in the discussion and assessment of the iron enrichment of foods dealt with in Section V and VI.

As has been clearly shown in tests on animals and man, the soluble iron salts, preferably of the ferrous valence, are most absorbable, so also it has been found that ferrous sulfate, among the iron sources most used in the fortification of foods exhibits the greatest bioavailability. On the basis of this property it is eminently suitable and, where it can be used, it is the enriching agent of choice. However, ferrous sulfate, in its present commercial form, has been found incompatible with storage stability and functional properties of certain food products, and other iron compounds of lesser bioavailability have been substituted. These latter substances have been finely divided iron powders and two insoluble salts of iron, ferric orthophosphate and sodium iron pyrophosphate.

The crux of the enrichment problem is the decision of how much bioavailability may be sacrificed to obtain product stability. This is specifically discussed in Section VI.

Appendix A deals with the effects of the methods of manufacture and the physical characteristics of elemental iron powders on their bioavailability. Appendix B discusses the development of an official assay for determining relative bioavailability of iron sources. Both subjects are of great importance in the future development of the iron enrichment program.

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I. INTRODUCTION

Medical interest in iron is very old. In Greek mythology iron was considered to be a gift from the god Mars and, in addition to its utility in the fabrication of weapons, its use as a medicament in the treatment of weakness or general debility long antedated knowledge of its physiological functions. Probably, the earliest iron-containing "tonics" were made from rust or iron filings which were steeped in wine to solubilize the iron in some degree (Diamond, 1970). Hahn (1937) states the Sydenham in 1661 and, later, others accurately described chlorosis and recommended its treatment with a variety of complicated prescriptions many of which contained iron. Even when Pierre Blaud in 1831 introduced his famous pill composed of ferrous carbonate, and recommended a daily intake of several grams of this iron salt there was no general agreement as to the underlying cause of chlorosis or proof of the value of this form of iron in its treatment. Considering the empirical basis of Blaud's therapy and the lack of basic physiological knowledge of iron metabolism at that time, this is understandable. It was almost one hundred years after Blaud's contribution before the value of inorganic iron salts was fully established as a treatment for hypochromic anemia due to iron deficiency.

In beginning a discussion of the bioavailability of iron compounds and the factors which influence their utility in the enrichment of foods, it will be of value to review briefly the changing concepts as to the form in which iron is absorbed held during the latter part of the 19th and the first half of the present century. In addition, the different procedures and tests for measuring iron absorption from the intestinal tract will be reviewed.

It must be kept in mind that at least two separate groups of research workers, clinicians and nutrition scientists, have been interested in iron metabolism and each has contributed to our knowledge of it. Basically, clinicians are concerned with the diagnosis and cure of diseases related to iron metabolism; nutrition scientists regard iron as an essential nutrient and are concerned with the amount and the form of iron in different food stuffs, the iron requirements of different segments of the population and the adequacy of available food stuffs in meeting these requirements. The clinician tends to prescribe therapeutic doses of iron to be taken separate from food while the nutritionist is concerned with a smaller daily intake which will prevent the appearance of deficiency. Each approach has complemented the other.

II. CONCEPTS AS TO THE FORM IN WHICH IRON IS ABSORBED FROM THE DIGESTIVE TRACT

The 19th century, especially the second half, was a fruitful period for exploring and understanding animal physiology, and especially the relation of food constituents to compounds found to be involved in various metabolic processes. It had long been known that iron was a normal constituent of the animal body and its presence in the ash of the blood and, in particular, in the blood corpuscles had been demonstrated. Toward the end of the 19th, and continuing well into the present century, it was a much debated question as to whether the body could absorb and utilize inorganic iron salts or must obtain it from complex organic compounds similar to those in which it occurs in the animal body. The most influential proponents of the need for organic forms of iron and the ineffectiveness of simple iron salts in the diet or as therapeutic hematinics were G. von Bunge (McCay, 1953) and his students, including Emil Abderhalden. Both von Bunge and Abderhalden were authors of influential texts on physiological chemistry which were available in this country in English translations.

As pointed out by McCay (1953), in his biography of von Bunge, the latter came honestly by his reputation as an expert in iron metabolism. Trained first as a chemist and later as a physician, he was appointed professor of physiology in Basel, Switzerland, in 1885. Even in his student days before receiving his degree in chemistry he became interested in the composition of blood and milk. He is credited with being the first to recognize that milk has a very low iron content, and he speculated as to how mammalian young maintained their hemoglobin level during the nursing period. The answer he found in the demonstration that the young are born with extra stores of iron in their bodies, particularly in the liver. The preparation and analysis of hemoglobin from the blood of several animal species by him and his students yielded the presently accepted value of 0.34 percent as the iron content of hemoglobin. Von Bunge also undertook the isolation of what he considered to be the key organic iron compound of eggs, which he called "hematogen". This he believed to be the precursor of hemoglobin in the chick.

In addition to analytical procedures for determining iron, attempts were made in von Bunge's laboratory to prepare low-iron diets for experimental animals in the hope that the absorption of iron might be studied. Some of the diets were based on milk and rice, both of which were known to be low in iron; others were based on dried horse serum, purified lard

and a special mineral mixture. Regardless of the diet or whether dogs or mice were used, the animals did not survive long enough to determine the value of specific iron supplements except that the mice receiving a supplement of egg yolk survived somewhat longer. No greater success attended the efforts of other laboratories which at that time attempted to resolve the question of organic versus inorganic iron.

It was not until the late 1920s that a biological test of sufficient precision was developed to permit easy assessment of the value of any iron compound in the repair of iron deficiency anemia. The Wisconsin Alumni Research Foundation workers (Hart *et al.*, 1928; Waddell *et al.*, 1928) showed that the young rat, weaned at around three weeks of age and thereafter restricted to a diet of fresh whole cow's milk, developed a profound anemia in three to four weeks. This anemia did not respond to iron alone, as measured by hemoglobin increase, but in the presence of a small supplement of copper, an element for which no nutritional requirement had previously been shown, the response to iron salts was dramatic. Thus an anemic rat about seven weeks old weighing between 70 and 80 g with a hemoglobin level of less than 4 g per 100 ml of blood, when given a daily supplement of 0.05 mg Cu and 0.5 mg Fe, would in eight weeks increase its body weight to between 160 and 170 g and its hemoglobin to 15-16 g per 100 ml.

The extension of these observations on rats to other experimental animals (chickens, pigs, mice) and to secondary anemias in human subjects has been reviewed by Elvehjem (1932). Wherever and to the extent that the anemia was due to iron deficiency, simple iron salts (often ferric chloride) given by mouth were found to be highly effective in its treatment. Not all nutritional anemias involve copper deficiency because this element is found in small amounts in most foods (milk is usually low unless it is processed in copper equipment) and indeed copper was found to be an impurity in many "pure" iron compounds.

With the above findings effectively disproving the long-taught concept that iron in simple iron salts was not absorbed from the digestive tract and hence of no therapeutic value, the Wisconsin Alumni Research Foundation laboratories (Elvehjem, 1932) undertook to compare the utility of iron in "organic combination" with that in ferric chloride. It must be realized that "organic iron" had never been well defined and there had been a tendency to equate it with food iron. By the late 1920s it was known that plant and animal food materials contained both inorganic and organic forms of iron. Of the latter the best known was hemoglobin or simpler heme compounds found in plant or animal cells which contain cytochrome enzymes. The material used by the Wisconsin Alumni Research Foundation workers was hemin (synonymous with heme) of known iron content crystallized from horse blood.

Using anemic rats maintained on the milk diet, the growth and hemoglobin responses to daily doses of 0.5 mg Fe from ferric chloride and from hemin were compared. In the absence of the copper supplement neither iron compound was effective; in the presence of 0.05 mg Cu the usual pronounced response to the ferric chloride was observed, while that from the hemin was only partial. The response to the hemin was such as to suggest that only a small percentage of its iron content was available; enough to increase the levels of hemoglobin from around 3 g to 7 or 8 g per 100 ml of blood during the first three-four weeks but falling lower than that as the animals continued on this regime. The iron content of the livers of the rats on the two supplements confirmed the hemoglobin values, those on the ferric chloride containing about five-fold the amount found in those on the hemin. Elvehjem in interpreting these findings made the statement: "These results point rather strongly to the fact that all iron compounds must be broken down into inorganic salts before the iron can be assimilated."

The above statement may be regarded, not only as putting an end to the inorganic versus organic iron controversy which had existed for half a century, but also as an expression of what was believed for the following quarter century to be the physiological facts as to the absorption of iron from the digestive tract. It is somewhat ironic that, in establishing the value of inorganic iron salts, the value of the truly organic form, such as that in heme, should have been inadvertantly downgraded. Whatever the reasons for the relative inactivity of Elvehjem's hemin preparation, his results were not representative of the values of foods containing heme iron or for hemoglobin preparations in the treatment of human iron deficiency established in the late 1950s and early 1960s.

In the interim, however, it was commonly held that only that iron in the food which could be liberated in ionic form by the digestive enzymes was available for absorption. Granick (1954) in a review of iron metabolism made the very positive statement: "The iron of iron porphyrins, such as the iron porphyrin of hemoglobin, is not available since iron porphyrins cannot be split in the gut to release the iron." Other active workers (Bothwell *et al.*, 1958; Moore and Dubach, 1956) reflect much the same belief with statements such as: "The human absorbs iron mostly, if not entirely, in its ferrous form."

That it took 25 or more years after Elvehjem's publication (1932) to appreciate the unique place of heme compounds in food as sources of iron is fully understandable for the following reasons. Most of the workers in this field during that time were clinically oriented and the focus of their attention was on therapeutic doses of iron for which purpose

the iron salts were much more useful, permitting larger doses of iron and more prompt therapeutic response in subjects with hypochromic anemia. Also newer and more precise methods had to be developed for measuring the amount of iron absorbed from the intestinal tract and the many factors which influence this important metabolic step. Without these improved methods based on radioiron isotopes it was not possible to explore and explain the unique position occupied by heme iron in human foods.

In coming to a fuller understanding of the value of certain foods, particularly those of animal origin, note must be taken of the results of nutritional research on normal human subjects in which the addition of beef to the diet had a favorable effect on iron balance (Johnston *et al.*, 1948; Leverton, 1941; McMillan and Johnston, 1951). Such findings could not be interpreted satisfactorily until techniques for the radioisotopic labeling of the iron in individual foods and in hemoglobin itself had been developed. The absorption of iron from many individual foods, including that from meat and from hemoglobin, has been reviewed by Layrisse and Martinez-Torres (1971) and it is now known that the bioavailability of the iron in foods of animal origin is greater than that from foods of plant origin. It is fully established (Callendar *et al.*, 1957; Turnball *et al.*, 1962) that this superiority is due to the fact that a large fraction of the iron in the former is present as hemoglobin and myoglobin in which the iron is bound as a metalloporphyrin (heme) complex. In the intestine the globin is split off by proteolytic enzymes and the iron containing heme is absorbed as such. While in the iron-porphyrin combination, the iron is unaffected by conditions in the intestinal tract (presence of food, soluble phytates, phosphates, or iron chelaters) which inhibit the absorption of inorganic iron. Such organic sources of iron in the food permit greater and more uniform absorption. Thus the present concept of the iron in a diet of mixed foods is that it may be regarded as belonging to one of two iron pools: heme and nonheme (Cook *et al.*, 1972; Layrisse and Martinez-Torres, 1972). These terms must now be substituted for the older, less precise, designations, organic and inorganic. In today's nomenclature the only organic iron is that in heme combination; all else is inorganic or nonheme, even including that found as hemosiderin or ferritin.

A guess may be ventured as to why Elvehjem's (1932) hemin was so poorly utilized as a source of iron for anemic rats. In the laboratory preparation of hemin from hemoglobin it has been found that there are opportunities for oxidation and/or polymerization of the former (Conrad *et al.*, 1966a). Apparently the amino acid chains of hemoglobin have a stabilizing influence on the steric structure of the heme prosthetic group. Very little absorption by human subjects was observed from purified acid-washed dialysates from incubated mixtures of labeled

hemoglobin and crude trypsin. However, large quantities of radioiron were absorbed from alkaline dialysates contaminated with globin degradation products (Conrad *et al.*, 1966a). Likewise the addition of niacin to test doses of heme decreased polymeric aggregation of heme and increased the absorption of heme-iron. Had Elvehjem fed a solution of hemoglobin rather than the isolated hemin he might have obtained more response to the "organic" iron. On the other hand, Conrad *et al.* (1966b) have noted that rats, mice and hamsters absorb very little iron from hemoglobin preparations. Therefore it is unclear as to the true reason for the poor response by rats to the hemin used by Elvehjem (1932).

III. METHODS FOR MEASURING ABSORPTION OF IRON FROM THE GASTROINTESTINAL TRACT

The very limited capacity of the human body, and that of most animals, to excrete iron, not really appreciated until the late 1930s (McCance and Widdowson, 1937, 1938), emphasized the importance of absorption as the chief regulator of the iron content of the body. Hence, the continuing interest in this metabolic step, in its measurement and in assessing the factors which influence it, can be appreciated.

Historically, the procedure which is now referred to as the hemoglobin repletion method, was the first to be used in what we may regard as the modern exploration of iron metabolism. Early examples of this were the Wisconsin Alumni Research Foundation experiments with anemic rats and those of Whipple and Robscheit-Robbins (1925; Robscheit-Robbins and Whipple, 1925) with dogs rendered anemic by repeated bleeding. However, this method will be treated more fully later and those procedures more suitable for application to human subjects will be considered first.

A. CHEMICAL BALANCE METHOD

Theoretically the difference between the amount of iron taken in by mouth and that excreted in the feces will provide a measure of the amount absorbed from the intestinal tract. Between 1930 and 1940 there were a number of such clinical studies of iron retention based on the chemical determination of iron in the ingesta and in the feces. Perhaps the most successful were those done with infants where it was easier to control fecal collections and the diet was largely milk. The technical difficulties were great, the most obvious being the quantitative collection of feces, the analytical care required to avoid iron contamination and to determine the small difference between intake and outgo, and the day to day variation in absorption requiring collection periods covering an adequate number of days.

The method had its greatest utility when the iron intake was limited to that contained in foods. Nutrition workers continued to use it in assessing the adequacy of representative diets and in demonstrating the value of special food items in improving iron balance (Johnston *et al.*, 1948; Leverton, 1941; McMillan and Johnston, 1951). An excellent summary and a critical review of the published work on iron balance,

both by chemical determination and by the following radioiron isotope method is provided by Josephs (1958).

B. RADIOIRON BALANCE METHOD

The utilization of radioactive isotopes of iron for studying iron absorption and other aspects of iron metabolism was first applied in the late 1930s (Hahn *et al.*, 1939). Since that time radiolabeled iron in inorganic salts or in intrinsically labeled food stuffs has been of the greatest importance in the development of present knowledge of iron metabolism.

As applied to iron balance studies it is generally agreed that the radioiron method is more accurate than the chemical method in that it is more specific and involves simpler analytical techniques (Bothwell and Finch, 1962). It does not, however, obviate the prolonged feces collections and their quantitative sampling, except for those few laboratories with whole-body counters. In the latter cases the difference between a count at two hours and one nine days after ingestion of the iron label can be taken as a measure of the iron absorbed. Of course, it cannot be lost sight of that radiolabel counting, by whatever method, also has inborn errors. Bothwell and Finch (1962) point out that the limited amount of iron absorbed by normal subjects, those for whom the balance methods are most appropriate, leads to a difference between intake and excretion which is of about the same order of magnitude as the inherent error of either chemical or radioiron procedure.

C. PLASMA IRON ABSORPTION CURVES

When it was realized that iron from the intestinal tract was absorbed into the blood plasma another "absorption" test was devised. It was found that by administering by mouth rather large doses of iron in the form of various salts to fasting subjects and by withdrawing blood samples over the following four to six hours for plasma iron determinations, a curve could be constructed showing the initial increase and later decline of plasma iron content. The similarity of these curves to those of the familiar glucose tolerance tests resulted in their being referred to quite often as plasma iron tolerance tests.

It was obvious that as measures of absorption such tests had to be interpreted with caution. The level to which the plasma iron rises and the rate of its decline are influenced by various factors; not only rate and amount of absorption but also the amount of unsaturated transferrin in the plasma, and the rate of the removal of iron from the plasma by other tissues. Nevertheless, this approach was extensively applied at one time and under standardized conditions useful information was acquired.

As an example, the work of Moore and his associates (1939) may be cited. These workers, using dogs and human subjects, obtained convincing evidence of the greater absorbability of the iron in ferrous form as contrasted with that in ferric form. In the majority of cases, the plasma iron absorption curves were higher following the ingestion of ferrous than of ferric salts. When, however, the latter were given with rather large amounts of ascorbic acid or other reducing agent the plasma iron responses were uniformly as good as those produced by ferrous salts of the same anion. They found no increase in plasma iron levels after the administration of the insoluble ferrous of ferric phosphate (NB, a flat curve does not exclude some absorption). Also, when subjects were permitted to take food just prior to, or immediately following, the ingestion of the iron salts there was frequently observed a smaller rise in the plasma iron than occurred under fasting conditions. This leads to a final comment on plasma iron absorption (or tolerance) tests: they cannot be regarded as physiological measurements because large (unphysiological, usually more than 50 mg) doses of iron must be employed. The amounts of iron absorbed from ordinary diets do not affect the plasma iron level.

D. PLASMA RADIOIRON ABSORPTION TEST

This method differs from that described above only to the extent that radiolabeled iron is administered and the "absorption" is measured in terms of radioactivity rather than by chemical determination. The major advantage of this method is that physiological amounts of iron can be employed because of the much greater sensitivity in methods for detecting the radiolabel.

E. RED BLOOD CELL RADIOIRON AS A MEASURE OF ABSORPTION

1. Use of Single Isotope

It was established early by several workers that iron introduced into the body was, in very large measure, utilized in the production of hemoglobin and appeared in the circulating red cells 10 to 14 days after administration. With the availability of radioactive iron this became the basis of the simplest and most commonly used method for determining the absorption of iron from the intestinal tract. From a practical point of view the method is simple. The radiolabeled iron is fed by mouth, a sample being retained for use later as a standard. Two weeks later a blood sample is collected, the red cells isolated, washed, and sampled and this and the standard are analyzed for radioactivity in a suitable counter.

This method relies on the assumptions that the iron in the red cell mass is in the form of hemoglobin, that it does not exchange with iron in the plasma, and that the total blood volume is known. The first assumption is well substantiated by abundant evidence that there is no measurable exchange between the iron of hemoglobin and the plasma during the life of the red cell; if it is necessary to have an accurate value for total blood volume this may be determined by one of several methods (such as by ^{51}Cr). In many cases a calculated blood volume, based on body weight, is adequate.

Because the erythropoeitic marrow is the most active of the body tissues as concerns iron turn-over, it is found that iron absorbed from the intestinal tract is, in very large measure, used for the formation of hemoglobin. Even in normal individuals as much as 80 to 90 percent of the absorbed iron may be found in the circulating red cells 10 to 14 days after ingestion; in iron deficiency anemia the utilization may approach 100 percent. On the other hand, disease states which depress marrow activity reduce the amount of iron incorporated into red cells and hence a greater proportion of the absorbed iron may be diverted to other pools. These situations must be taken into account in using the single radioiron isotope to measure the total amount of iron absorbed from the gut. Usually the greatest use of this method is in comparative studies in the same or similar subjects, where the effects of gastrointestinal conditions on absorption of iron are assessed, and the assumption of a certain percentage utilization in the formation of hemoglobin involves no errors in the interpretation of results.

2. Double Isotope Method

The basis of this technique, as first proposed (Saylor and Finch, 1953), is to administer one isotope (^{55}Fe, usually) by mouth and at the same time a tracer quantity of the second isotope (^{59}Fe), bound to transferrin, is given intravenously. The amount of iron and its specific activity contained in each dose, is known and two weeks later a sample of blood is drawn and by differential counting the amount of each isotope is determined.

The basic assumption of this procedure is that the iron absorbed from the gut is transported as plasma transferrin to the body tissues and its subsequent distribution is in every way similar to that of the iron given intravenously. Thus the fraction of the total injected label found later in the red cells of the blood sample is assumed to represent the fraction of the other label absorbed from the total given by mouth. In this way the percentage of absorption of the latter can be calculated. This calculation automatically corrects for tissue distribution and obviates any need to estimate blood volume. This method is considered to be the most accurate and the most convenient of the various isotopic methods for measuring iron absorption which has been developed (Bothwell and Finch, 1962).

Variations in the manner of using the two radioactive isotopes of iron for measuring intestinal absorption have been introduced. Thus Brice and Hallberg (1962a) have applied this technique in the following manner. Two different compounds of iron (e.g., ferrous sulfate and ferric sulfate) were labeled, one with ^{55}Fe and the other with ^{59}Fe. Doses of both salts containing equivalent amounts of iron, and each of known specific activity, were administered by mouth to human subjects over a 10-day period; the one label being given on the odd-numbered days and the other on the even-numbered days. The object of spreading the total dose of each of the labeled iron compounds over five separate doses was to reduce the error due to daily variations in intestinal absorption. Further to reduce systematic errors the subjects were divided between those who received ^{55}Fe as the first dose and those who received ^{59}Fe as the first dose. From analyses of ^{55}Fe and ^{59}Fe activities in a blood sample drawn two weeks after the last oral dose the mean absorption ratio of the two isotopes was calculated. It will be noted that each subject served as his own control.

F. HEMOGLOBIN REPLETION METHOD

The basis of this procedure is the measurement of the response in the hemoglobin content of the blood (or the increase in hemotocrit volume) to graded doses of iron in experimental animals rendered severly anemic by iron-deficient diets. Although not usually thought of as a method for measuring the intestinal absorption of iron, it is in fact a quite precise assay procedure by which the bioavailability of iron from different sources may be determined. Like most bioassay methods it is customary to include a standard reference iron source in each test and to express the potency of each "unknown" as a percentage of the reference standard, the responses to which are arbitrarily set at 100. By the use of radiolabeled iron sources and suitable counting techniques it is possible to obtain results in terms of amount of iron absorbed from the intestinal tract, as demonstrated by Amine and Hegsted (1971). The latter used a whole body counter in experiments with rats, accepting the difference between the counts at two hours and at nine days after ingestion of the sample as a measure of iron retention.

The method derives from the work of the Wisconsin Alumni Research Foundation, as mentioned (Waddell *et al.*, 1928) showing that a severe anemia develops in weanling rats and other experimental animals (Elvehjem, 1932) restricted to a diet of cow's milk. In its early application, "mineralized" milk (cow's milk supplemented with small additions of copper and manganese) was used as the basal diet. Later, because of greater convenience, basal diets based on dried milk or purified casein and glucose or other carbohydrate source plus low-iron mineral and vitamin supplements were substituted (Amine and Hegsted, 1971; Pla and Fritz, 1970). Any nutritionally well balanced diet, sufficiently low in iron (less than 10 ppm), is suitable. The method has been applied in both a prophylactic and in a curative manner.

The hemoglobin repletion method has been of great value in assessing various foods as to the availability of their iron content. Summerfeldt (1935) showed that the iron contained in common foods used in the feeding of infants and children which could be solubilized by a three-hour digestion in an artificial gastric juice was a much better measure of their value in regenerating hemoglobin in anemic rats than was the total iron of the same foods. Other results of the early use of the method have been reviewed by Bing (1972).

It is of interest to note that following the first flour and bread enrichment program (Federal Register, 1941) this method was used to assay the availability of the iron supplements in use for that purpose

(Blumberg and Arnold, 1947; Freeman and Burrill, 1945; Nakamura and Mitchell, 1943; Street, 1943). Details of these assays are discussed more fully in a later section. Recently, there has been renewed interest in applying the method in the assay of a much wider list of iron-containing substances and in the factors which influence the bioavailability of their iron content (Amine *et al.*, 1972; Fritz *et al.*, 1970; Morris and Green, 1972). All in all, the procedure is the most useful of animal assays for this purpose and it has been proposed as an official method of the Association of Official Analytical Chemists (AOAC) (Pla and Fritz, 1970). The results secured have been reasonably in consonance with those obtained from experiments with human subjects.

IV. REVIEW OF FACTORS INFLUENCING ABSORPTION OF IRON FROM THE GASTROINTESTINAL TRACT

There are many factors which influence the absorption of iron from the gastrointestinal tract, not all of which have equal significance in relation to the problems of the enrichment of various foods with iron compounds. Those factors which cannot be assessed or controlled in the general public are discussed in a general way; those which have a direct bearing on the problem of food enrichment are discussed more fully.

A. PHYSIOLOGICAL FACTORS

Both the status of body iron stores and the level of activity of the erythropoietic marrow have been shown to influence iron absorption; in iron deficiency, the increased absorption of iron from both iron salts and from food has been fully established (Bothwell *et al.*, 1958; Pirzio-Biroli *et al.*, 1958). It is of particular interest to note the results of observations on two groups of normal human males, one group having the iron stores reduced by phlebotomy and the other having the iron stores enlarged by intramuscular injection of iron dextran (Pirzio-Biroli and Finch, 1960). During the study, which extended over a period of 753 days, all the subjects ate a normal diet without medication or supplements containing iron. Beginning on day 188 and at intervals thereafter hematocrit values determined on the subjects showed no significant difference between the two groups. Plasma iron values and total iron binding capacities were also determined at intervals and although the average values for both of these indices were slightly higher in the iron-depleted group they were within the normal range and without significance as indicators of the status of the iron stores. On the other hand, iron absorption from the intestine, measured by the double isotope method on three occasions between days 196 and 748, showed in every case greater absorption by the iron-depleted group (average 19 percent versus 4 percent).

These results indicate that individuals with diminished iron stores without anemia may exhibit enhanced iron absorption similar to that seen when both iron deficiency and anemia are present. They also are the basis for the belief that both the status of the iron stores and the activity of the erythropoietic marrow influence the absorptive capacity of the intestinal mucosa by some physiological mechanism which is still unknown. The greater iron absorption usually exhibited by women

as compared with men is best explained by the fact that their iron stores are less likely to be replete than those of men.

B. EFFECT OF IRON VALENCE

As early as the 1930s clinicians were aware of the efficacy of ferrous sulfate in the treatment of iron-deficiency anemia and had found that lower daily doses of this form of iron were required than of others such as reduced iron, ferric ammonium citrate, and other complex iron preparations (Reimann and Fritsch, 1930; Witts, 1936). Fowler and Barer (1939), in discussing the then current methods of treating iron-deficiency anemia, reported that the recommended daily doses of the following commonly used preparations were: iron and ammonium citrates 6 g (1000 mg Fe); reduced iron 3 g (2700 mg Fe); ferrous carbonate 4 g (360 mg Fe); ferrous sulfate 1 g (200 mg Fe). Although the different amounts of iron contributed by these doses reflect, in some degree, the differences in efficacy among them, it must also be remembered that the dose was influenced by the degree of "intolerance" (abdominal cramps, diarrhea) suffered by some patients. Fowler and Barer in referring to ferrous carbonate advised that the pills of this compound be freshly prepared because oxidation to an insoluble form might occur, or, by becoming dry and hard, would not disintegrate in the digestive tract.

In addition to clinical observations there has been experimental evidence by many investigators that human subjects absorb ferrous iron much more readily than ferric iron (Hahn *et al.*, 1945; Henley *et al.*, 1956; Moore *et al.*, 1939, 1944). The most precise comparisons between the two valence forms have been obtained by administering the doses of iron after an overnight fast and withholding food for several hours after the dose. Absorption was assessed by determining the increase in serum iron at intervals after dosing (Moore *et al.*, 1939); by using iron salts tagged with ^{59}Fe and later measuring the radioactivity in the red blood cells (Hahn *et al.*, 1945; Moore *et al.*, 1944); or by a combination of both of these methods (Henley *et al.*, 1956). In many of these experiments the two valence forms of iron were tested at different times on the same subject. Both normal and iron-deficient subjects were used.

Comparisons were made between for example, ferrous and ferric chloride, ferrous ammonium sulfate and ferric ammonium sulfate, and between ferrous sulfate and other ferrous salts, such as ferrous chloride, ferrous ascorbate, ferrous gluconate, ferrous carbonate and

ferrous ammonium sulfate. All of these ferous salts produced serum iron increases comparable with that produced by ferrous sulfate and the ferrous salt was more effective than the ferric salt of the same anion. However, when ferric chloride was given in the presence of a large supplement of ascorbic acid or other reducing agent, serum iron response was as good as that obtained from ferrous chloride. On the other hand, neither of the relatively insoluble ferrous or ferric orthophosphates brought about appreciable changes in serum iron values (Moore *et al.*, 1939) indicating that degree of ionization of the iron supplement is critical for its absorption. In studies made on patients with severe degrees of hypochromic microcytic anemia the absorption of iron from ferrous chloride was from 2 to 15 times that absorbed from ferric chloride (Moore *et al.*, 1944).

Brise and Hallberg (1962b) applied their method of using the two isotopes of iron (see p 21) to a study of the absorption of 14 different iron salts which might be of therapeutic value in the treatment of iron deficiency. In a total of 68 subjects, each of whom received ferrous sulfate as the reference standard and one of the other 13 compounds, the absorption of the latter compounds was expressed as a percentage of that of ferrous sulfate with the results shown in Figure 1. All of the iron compounds were given as solutions, each containing 30 mg of Fe, after an overnight fast and without food or water for two hours after the dose. In each case the ferrous sulfate, tagged with either ^{55}Fe or ^{59}Fe, was administered on alternate days during the 10 days of dosing, and the other iron compound, tagged with the alternate isotope was administered on the other five days. The relative absorption of each form of iron was calculated from the activity of each isotope found in the red cells of a blood sample taken two weeks after the last dose. Thus, each line in Figure 1 represents a comparison, in the same individual, of the absorption of one of the other iron compounds with ferrous sulfate.

It is evident from Figure 1 that the absorbability of iron from different iron compounds differs markedly. Under the conditions of this method of testing, all ferric compounds were less well absorbed than were the ferrous compounds even though there were significant differences among the latter. In particular, the iron in ferrous citrate, ferrous tartrate, and ferrous pyrophosphate[1] was less well absorbed

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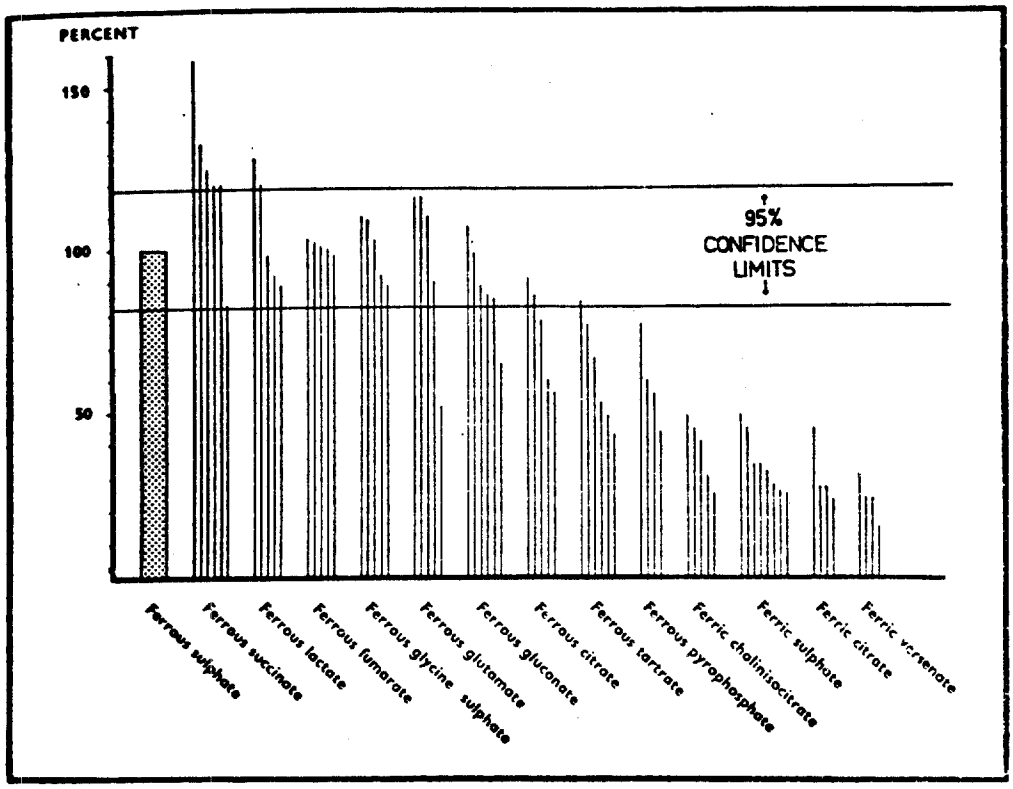
[1] There is reason to doubt the identity of this compound. Chemical handbooks do not describe a ferrous pyrophosphate; the authors made no mention of the source or the nature of the compound actually used. One may speculate that it might have been ferrous orthophosphate, but this also is doubtful because of its low solubility.

FIGURE 1

ABSORPTION OF IRON FROM DIFFERENT COMPOUNDS
COMPARED WITH THAT FROM
FERROUS SULFATE



From Brise and Hallberg (1962b).

than that from the other ferrous salts. Brise and Hallberg (1962b) explain this on the basis that in these three compounds "an appreciable part of the iron exists as complex ions." Similarly, the poor absorbability of ferric salts in general and, in the present case, particularly that of ferric versenate (ferric complex formed with ethylenediaminetetraacetic acid is best explained by their propensity for form very strongly bound complexes (chelates) from which the iron is slowly and poorly liberated in ionic form. Conrad (1970) has discussed the physicochemical characteristics of the two valence forms of iron and the comparative solubilities of their salts over the pH range of the gastrointestinal tract. For example, iron is precipitated from ferric chloride as the pH approaches 5 while the ferrous chloride remains soluble up to pH 8-9. Considerations such as these provide the most convincing explanation of the beneficial effect of ascorbic acid, or other reducing substance, on the absorption of iron from the gut; a phenomenon which has been repeatedly observed.

C. EFFECT OF FOOD ON ABSORPTION OF IRON AND THE ABSORPTION OF FOOD IRON.

1. Effect of Food on Absorption of Therapeutic Doses of Iron

In the investigations reviewed above the iron compounds were administered after a fast of several hours (generally overnight) and without interference from ingested food for several hours after the dose. Moore *et al.* (1939) in studying iron absorption by the increase in serum iron noted that if subjects were permitted to take food just prior to, or immediately following, the dose of iron, there was observed quite frequently a smaller rise in the serum iron than occurred under fasting conditions. Hahn *et al.* (1943) in experiments with normal and anemic dogs given radioactive iron noted differences in the plasma radioiron absorption curves between iron given to fasting dogs and that given with food. In the former situation the peak of absorption occurred at one to two hours but in the latter the peak was delayed several hours.

Brise (1962), using the double isotope method discussed above, studied the effect of a light meal on the absorption of iron from a therapeutic dose. Nine subjects were used, three of whom were considered to be normal as regards their iron metabolism while six were known to be blood donors. During a 10-day period each subject received each morning a solution of ferrous sulfate equivalent to 30 mg Fe labeled with either ^{55}Fe or ^{59}Fe. On each of five alternate days when the iron was labeled with one of the isotopes the dose was given after an overnight fast and without food or water for an additional two hours. On the

other five alternate days the iron dose labeled with the other isotope was given one-half hour after a light meal. This latter consisted of one glass milk, one cheese sandwich (white bread), one cup coffee (no cream or sugar). The relative activity of the two isotopes in the red cells of a blood sample taken two weeks after the last dose permitted a calculation of the effect of the meal.

In each of the nine subjects the absorption of iron given after the meal was less, the mean value being 56 percent of that given in the fasting state. It is of interest to note that the percentage effect of the meal on the absorption of iron by the normal subjects was greater than on that by the blood donors.

2. Absorption of Food Iron

a. Nonheme iron. In a discussion of factors which influence the absorption of nonheme iron from individual foods or mixtures of foods it is instructive to review briefly some of the older nutritional literature on mineral metabolism including that of phosphorus. Cox *et al.* (1931) found that when they fed guinea pigs or rabbits diets containing soluble aluminum salts there was a pronounced lowering of the inorganic phosphorus of the blood plasma and a reduction in the calcium, phosphorus, and total ash of the bones. Ferric salts produced a similar but less marked effect. The addition of monosodium phosphate to these diets, equivalent to the aluminum or ferric salts, completely prevented the untoward effects on the bone and on the plasma phosphorus. Similarly, Deobald and Elvehjem (1935) reported that day-old chicks receiving a normal ration to which was added large amounts of soluble iron or aluminum salts developed severe rickets in one to two weeks. A definite drop in blood phosphorus was observed as early as the fifth day. The addition to the diet of sodium acid phosphate in amounts sufficient to unite with the added metals allowed rapid growth and normal bond formation. These authors raised the question of possible danger of phosphorus deficiency to human patients from the high doses of iron used in the therapy of hypochromic anemias.

Beryllium rickets, described by Branion *et al.* (1931), was observed when the calcium carbonate of Steenbock's 2965 rachitogenic diet for rats was replaced by an equivalent amount of beryllium carbonate. The bone lesions of rickets and extremely low levels of inorganic phosphorus of the blood plasma were observed, but these changes were completely refractory to the administration of vitamin D or to direct irradiation of the animals with ultra-violet light, which was in contrast with the rickets produced by the regular rachitogenic diet.

Manganese rickets was produced in rats by the substitution of an equimolar amount of manganese carbonate for the calcium carbonate in a high-calcium, low-phosphorus rachitogenic diet, and also by the addition of manganese carbonate to a stock diet of optimal calcium and phosphorus content (Blumberg *et al.*, 1938). Indeed, these authors noted that the bone calcification, ordinarily observed when disodium phosphate was added to the rachitogenic diet, was manifestly retarded by the simultaneous addition of the carbonates of bismuth and calcium but even more so by the carbonates of iron, magnesium, strontium, and manganese.

In these investigations the basic nutritional error was the creation of a phosphorus deficiency. All of the cations mentioned, including iron, form insoluble phosphate salts and the evidence indicates that when present in relatively large amounts with border-line levels of phosphorus, the latter is so poorly absorbed from the digestive tract as to create a distinct deficiency. Obviously, in the context of this report, interest centers, not on cations such as beryllium, strontium, aluminum, etc., which are not found in significant amounts in human or animal diets, but on the iron. If iron can complex and render phosphorus unavailable, the converse is also true: phosphorus is capable of rendering iron relatively unavailable especially if the latter is present in limited amounts (Day and Stein, 1938). Certainly, phosphate salts of iron would not suggest themselves as suitable supplements for iron enrichment of foods.

Other examples of the dietary iron-phosphorus relationship have been reported. Hegsted *et al.* (1949) found that adult rats, fed a diet composed of corn grits and lard to which was added 2 percent of ferric citrate, absorbed excessive amounts of iron as judged by the amount found in their livers. The diet was patently deficient in several nutrients and the animals lost weight whether the iron was added or not. In searching for dietary supplements which might influence the absorption of the excess iron, it was found that the addition of a complete salt mixture was beneficial and that higher levels of phosphate salts in the mineral mixture or added alone to the diet was more effective. The authors concluded that the amount of iron deposited in the liver was inversely related to the phosphorus content of the diet.

It has often been postulated that phytates, which occur mainly in cereal foods, interfere with the absorption of iron by the formation of insoluble iron phytate during the process of digestion. There is little precise information as to the extent of such a reaction in mixed diets containing cereal products or to its nutritional significance. Early experiments by Rose and her associates (Bing, 1972) showed that the iron in wheat and its by-products was well utilized by young rats.

In human subjects Layrisse and Martinez-Torres (1971) found that the bioavailability of the iron in both wheat and corn compared favorably with that in the other foods of plant origin which they tested.

Sharpe *et al.* (1950) studied the effect of phytate and other food factors on iron absorption by adolescent boys. The phytate was that present in rolled oats which was fed as cooked cereal in four of the seven test meals which were eaten as breakfast. One of the meals (v) was water only. A preparation of sodium phytate was added to one meal to match the phytate found to be present in the rolled oats. The composition of the various meals is shown in Table 1. The iron content of all the meals was approximately the same (7.6 - 8.3 mg), present either in the food components or as ferric chloride added to the milk or water. This addition also carried one of the two radioiron isotopes, ^{55}Fe or ^{59}Fe. All meals were given between 7 and 8 a.m. after a fast from dinner the evening before and no other food was given until noon of the day of the meal. Absorption of iron from various meals was calculated from the radioactivity found in the red cells of a blood sample taken eight or more days after the test meal.

The results of this experiment are shown in Figure 2 where the percent of iron absorption is plotted against solids content of the test meals. The high absorption (26.9 percent) from the water alone (meal v) is explainable on the absence of interferring substances. Milk alone (meal ii) reduced the absorption by about one-third; which could not be due to phytate because the milk contained none. The combination of milk and rolled oats (meal iv) reduced the absorption of iron by two-thirds. Thus the interference from rolled oats was not more than that from milk. There was no correlation between the phytate content from rolled oats and the reduction in iron absorption. The effect of the sodium phytate added to milk (meal iii) had a profound effect in depressing iron absorption (1.74 percent). This may be explained on the basis that the sodium phytate was soluble and easily ionized, permitting the phytic acid to react with the iron in the milk to form a complex which was poorly dissociated and from which iron could not be absorbed. It is quite obvious that sodium phytate and the phytates which occur in oats are quite different compounds as regards their effect on food iron. There is reason to believe that most naturally-occurring phytates, such as those contained in cereals, are present as the mixed calcium and magnesium salt of phytic acid (phytin) and as such, because of poor solubility, are likely to be relatively inactive chemically during the process of digestion. The present experiment would indicate that such phytates may have a lesser effect on the iron of the diet, whether native or added by enrichment, than has been supposed in the past.

TABLE 1

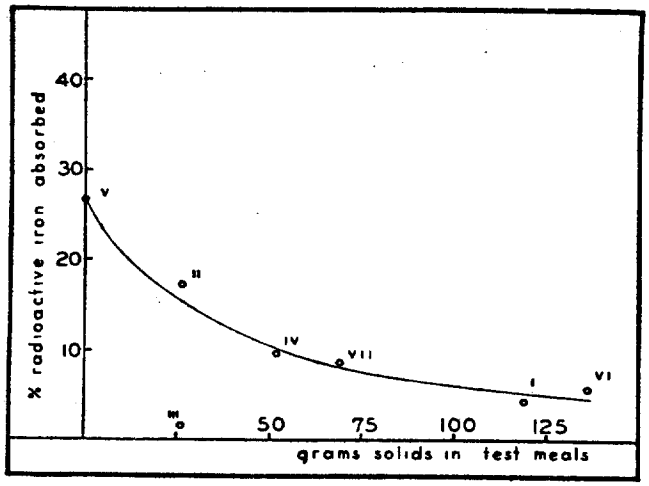
COMPOSITION OF TEST MEALS (GRAMS) USED IN MEASURING ABSORPTION OF IRON BY ADOLESCENT BOYS

Test Meal	I	II	III	IV	V	VI	VII
Milk	200	200	200	200		200	200
Water					200		
Cooked Rolled Oats	285			173		285	285
White Bread	34					56	
Egg Omelet	75					75	
Tomato Juice	150					150	
Sodium phytate	___	___	0.2	___	___	___	___
Total Wet Weight	744	200	200.2	373	200	766	485
Total Dry Weight (calculated)	119	26	26	52	0	136	69

(From Sharpe *et al.*, 1950).

FIGURE 2

RELATIONSHIP BETWEEN SOLIDS CONTENT OF TEST MEALS AND ABSORPTION OF RADIOACTIVE IRON



(From Sharpe *et al.*, 1950).

The inverse correlation of iron absorption with the solids content of the test meals as indicated in Figure 2, as interpreted by Sharpe *et al.* (1950) is a plausible and an attractive concept to explain much of the physical effect of food on the absorption of iron from the gut. It seems reasonable to speculate that, in comparison with a water solution of an iron salt, the presence of food solids has the physical effect of diluting the iron and reducing the concentration of iron atoms per unit of mass or volume of the ingesta. There would thus be fewer chances for iron atoms to come into contact with the absorbing surface of the duodenum and upper jejunum where iron is best absorbed. A corollary of the above is that the higher the iron content of a food, either intrinsic or added, the lesser will be the dilution effect of the food solids.

b. Heme iron. Reference has been made to the belief, prevailing until relatively recent years, that only that portion of the food iron which could be broken down during digestion into inorganic form was available for absorption; specifically that the iron in the porphyrin ring (heme) was not available.

Recent investigations have demonstrated that this concept was in error and have added important new facts concerning the mechanism of absorption of heme iron. Although inorganic sources of iron are the only substances used or likely to be used for the enrichment of foods, it is instructive to review the special characteristics of heme iron and the manner in which it is absorbed from the digestive tract. It is well recognized that meat is among the richest sources of iron in the human diet and that those populations which obtain an appreciable portion of their iron intake in this form are less likely to show evidence of iron deficiency. Layrisse *et al.* (1973) have found that animal protein in a mixed diet, in addition to being a source of heme iron, had a favorable effect on the absorption of inorganic fortification iron.

Experimental findings which support the resumé below as to the special characteristics of heme iron and its mode of absorption have been reported by: Bannerman (1965); Brown *et al.* (1968); Callendar *et al.* (1957); Conrad *et al.* (1966a, 1966b, 1967); Turnbull *et al.* (1962); Walsh *et al.* (1955); Weintraub *et al.* (1965, 1968).

The most useful preparation of heme iron is the hemoglobin from lysed red cells of an animal (sheep, rabbit, etc.) which previously had been injected with radiolabeled iron of high specific activity. At dosage levels of around 5 mg iron, hemoglobin iron was absorbed as well as, or better than, that of ferrous salts in normal human subjects. In iron-deficient subjects the absorption of hemoglobin iron increased but to an extent less than that of ferrous salts. Similarly, an increase in the dose had a lesser effect on the absorption of hemoglobin

iron that on that of the iron salt. On the other hand, the presence of food, the addition of sodium phytate to the food, or the presence of the iron-chelating agent, desferrioxamine, failed to inhibit the absorption of hemoglobin iron, in distinct contrast with their effect on the absorption of iron salts. Ascorbic acid had no beneficial effect on the absorption of iron from hemoglobin. Even when 100 mg Fe as ferrous sulfate was administered simultaneously with 5 mg hemoglobin iron the absorption of the latter was essentially the same as when fed alone, indicating that the two forms of iron do not compete with each other for absorption.

The above evidence is interpreted as indicating that the hemoglobin iron is absorbed into the mucosal cells in the form of the heme moiety, the action of the digestive enzymes extending only to the splitting off of the amino acid chains from the heme prosthetic group. In man, the heme is split into porphyrin and ionic iron within the intestinal mucosal cell, since only transferrin-bound iron has been found in the blood plasma during the period of absorption following a dose of heme iron. In certain animal species, such as the dog and the guinea pig, evidence has been obtained that an appreciable portion of the heme, as such, tranverses the mucosal cell and both heme and transferrin-bound iron may be identified in the blood of these species if the samples are obtained before the blood reaches the liver (e.g., from the common portal vein).

The absorption of heme iron from laboratory preparations such as hemin has routinely been poorer in both man and laboratory animals than from hemoglobin itself. This has been explained on the basis of the tendency of hemin to become insoluble at acid pH or to polymerize into macromolecules at alkaline pH, both of which militate against absorption. The amino acid chains of intact hemoglobin appear to have a stabilizing effect on the heme group during passage through the acid stomach and the splitting of the molecule by the proteolytic enzymes of the duodenum occurs at a time when the heme moiety will be immediately available for absorption.

D. SPECIES DIFFERENCES IN IRON ABSORPTION

The extensive literature on iron metabolism of the past fifty years has provided evidence that there are differences between man and certain other animal species in the way in which iron is handled physiologically. Most, but not all, of these differences occur in the absorption of the various forms of iron from the gastrointestinal tract.

Ferric chloride was one of the first preparations of pure iron used in early experiments for the repair of iron deficiency in the rat and in continued to be used as the standard iron supplement. No evidence was noted as to the relative efficacy of different soluble iron salts not to any difference related to the valence form of the iron. Because of the reported marked superiority of the ferrous forms of iron in the treatment of hypochromic anemia in man, Underwood (1938) undertook to compare suboptimal amounts of ferrous ammonium sulfate and ferric ammonium sulfate in the cure of severe iron deficiency in young growing rats. In two carefully controlled experiments in which the rate of growth and the rate of hemoglobin regeneration were compared over a period of five weeks, no difference between the two iron supplements was found. The deposition of iron in the liver determined at the end of the experiments was the same for both supplements.

Austoni and Greenberg (1940) in early experiments with radioactive iron noted no significant difference between the absorption by rats of iron from ferrous sulfate or from ferric chloride. Similarly, Street (1943) found both ferrous sulfate and ferric sulfate to be equally efficacious in the cure of iron deficiency in rats consuming a milk diet. Blumberg and Arnold (1947), in testing the efficacy of breads baked with the addition of ferrous sulfate, ferric chloride, or ferric orthophosphate, found that the breads containing ferrous sulfate and ferric chloride were equally efficacious in curing iron deficiency anemia in young rats; both of these sources possessed four to five times the efficacy of the bread containing ferric orthophosphate. The subsequent literature does not refute these findings.

Dogs seem to share, at least under certain circumstances, this same ability to utilize the iron of ferric salts equally as well as that of ferrous. In some of their early experiments Whipple and Robscheit-Robbins (1930), in testing the ability of iron salts to sustain hemoglobin production in dogs, regularly bled to maintain a low level of hemoglobin, reported that ferric chloride, ferric citrate, ferrous carbonate, ferrous sulfate, and ferrous amonium sulfate all produced similar results. These iron salts were added to the standard low-iron "salmon bread" on which the dogs were maintained and efficacy was judged by the amount of hemoglobin in the blood which they needed to withdraw each week to maintain the hemoglobin at the predetermined low level.

A more accurate and more sensitive method of assessing iron absorption in dogs was applied by Moore *et al.* (1944). Using a dose of 1 to 4 mg iron per kg body weight, solutions of radiolabeled ferric and ferrous chloride were administered at different times to the same dog in a fasting state. Absorption was calculated from the radioactivity

found in the red cells of a subsequently drawn blood sample. Normal and iron deficient animals were used. It was found that in the anemic dogs equal amounts of the two valence forms of iron were absorbed. In the nine comparisons made on the normal dogs one three showed equal absorption of the two forms of iron; six showed greater absorption of the ferrous iron. These findings suggest that dogs fall between man, who uniformly differentiates in favor of the ferrous iron, and the rat, which uniformly absorbs both forms equally, at least from soluble iron salts. How other species respond has not been determined. One may speculate that these differences reflect in some way the level of ascorbic acid in the tissues and in the gastrointestinal secretions of the different species. Man, requiring a dietary source of ascorbic acid, may be presumed to have lower levels of this reducing and iron-chelating agent in his secretions than those species which are capable of its physiological synthesis. Conrad (Heinrich, 1970) states that, even in man, both gastric juice and bile contain appreciable amounts of ascorbic acid.

Species differences have been noted in the absorption of the iron of hemoglobin or of the isolated hemin. Bothwell and Finch (1962) make the statement, "hemoglobin iron is about five times better absorbed in man than in the rat," without giving a literature reference in support of it. The work of Turnball *et al* (1962), of course, demonstrated the good absorption of this form of iron in man, and other subsequent publications (Conrad *et al.*, 1966a; Weintraub *et al.*, 1965) reported the poor absorption of hemoglobin iron in the rat and also in mice and hamsters. By raising rats on a diet containing hemoglobin as the only source of iron the animals were in a constant state of iron deficiency (Weintraub *et al.*, 1965). On the other hand, Bannerman (1965) found that the British rats which he used absorbed hemoglobin iron well over a range of doses. This marked difference in experimental findings suggests that there may be strain differences in rats.

Chappelle *et al.* (1955) have measured iron loss in mice under varying conditions and have found that a 10-fold range in iron excretion may exist. In the mouse, therefore, absorption and excretion rates would appear to be of the same magnitude in their capacity to regulate iron balance.

V. EXPERIENCE WITH IRON-ENRICHED CEREAL FOODS

A. EARLY HISTORY

From the beginning of the enrichment program the untoward effects of certain iron compounds on the stability of flour and flour-mixes was recognized. Tobey and Cathcart (1941) noted that the addition of iron to flour was more difficult than the addition of the vitamins. They stated that, if an iron salt were not entirely insoluble, it would cause adverse reactions, such as rancidity, in the flour. In the statement of the Council on Foods and Nutrition (1941) endorsing the principle of nutritionally improving, or enriching, flour and bread, it was reported that the flour milling industry had proposed the use of iron phytate, sodium iron pyrophosphate, and reduced iron[2] as sources of iron in enriched flour. The Council, at that time, reserved judgment as to the choice of iron compounds to be added because of lack of information on the assimilability of the iron, but observed that there would be no nutritional advantage in adding a compound, the iron of which would be unavailable. This problem has not been resolved in the years between 1941 and the present. Compounds are still being used in the enrichment of food products because of their light color and chemical inertness rather than for the bioavailability of their iron.

Immediately following the initiation of the enrichment program several assays on anemic rats were carried out on the iron compounds being used commercially. Nakamura and Mitchell (1943) compared the relative availability of the iron of ferric phytate, sodium iron pyrophosphate, and reduced iron with that of ferric chloride. The ferric phytate was found to be a poor source because its iron was only one-half as available as that in the standard. The iron of sodium iron pyrophosphate and of reduced iron was reported to be well utilized for hemoglobin regeneration. In the light of subsequent assays of sodium iron pyrophosphate these findings of Nakamura and Mitchell (1943) are surprising and one explanation could be that the basal diet used was not sufficiently low in iron content. The animals were not well depleted of their iron reserves and were started on test with hemoglobin levels much higher than is usual. The authors noted there was great variability in their results. Presumably because of these results with ferric phytate and

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[2] "Reduced iron" has been the name applied in this country to all forms of powdered iron used for food enrichment. See Appendix A of this report.

equally poor results in a test on human subjects (Moore *et al.*, 1943) this source of iron was little used in the enrichment program.

Street (1943) compared sodium iron pyrophosphate with ferrous sulfate in two tests with anemic rats. In one test the iron compounds were administered directly by mouth and in the second as dried breads to which the iron compounds had been added during baking. In both tests the iron of sodium iron pyrophosphate was "considerably less than 50%" as available as that of ferrous sulfate.

Freeman and Burrill (1945) compared sodium iron pyrophosphate, reduced iron, and sodium ferric orthophosphate with ferric chloride as supplements to a milk diet for anemic rats. In a second experiment these compounds and ferric orthophosphate were used as the enriching agents in five specially prepared dried breads fed as supplements to the milk diet. In a third, smaller experiment ferric chloride and sodium iron pyrophosphate were compared as to their efficacy in preventing anemia in weanling rats maintained on a milk diet. In the three experiments, results were judged not only on hemoglobin levels in the blood but also on the total iron content of the carcasses at the end of each test.

In summarizing their results, Freeman and Burrill (1945) used the following order of effectiveness as expressing the relative degree of iron retention and hemoglobin regeneration: ferric chloride > sodium ferric orthophosphate = ferric orthophosphate > reduced iron > sodium iron pyrophosphate. In reviewing the figures one can not disagree with this ranking but it should be noted in all three experiments the iron of sodium iron pyrophosphate was always less than 50 percent as effective as the iron of ferric chloride.[3]

A fourth bioassay, carefully designed and meticulously carried out, was reported by Blumberg and Arnold (1947). These authors noted that up to that time ferric orthophosphate was the form of iron most generally used when enrichment was effected at the bakery and decided to compare it with the more soluble ferrous sulfate known to be highly efficacious in clinical therapy. Three lots of bread were baked from the same batch

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[3]Sodium iron pyrophosphate, synonymous with sodium ferric pyrophosphate (Food Chemicals Codex, 1972) was supplied by Victor Chemical Works, Chicago, Illinois to Nakamura and Mitchell, to Street, and to Freeman and Burrill. Later Victor became part of the Stauffer Chemical Company, Westport, Connecticut, which is credited with supplying most, if indeed not all, of the sodium iron pyrophosphate used today in the iron enrichment of foods.

of flour with special enrichment mixtures which supplied the usual amounts of vitamins but varied with respect to iron. One lot of bread contained no added iron and was used for the negative control group of rats, a second lot contained ferric orthophosphate, and the third ferrous sulfate. The breads were air dried, ground, and analyzed for actual iron content. By suitable dilution with the negative control bread, a series of four levels of each of the supplemented breads was prepared which, in each case, made up 82 percent of the dry diet used for the repletion test. The rats had previously been well depleted on mineralized milk; all the groups had average hemoglobin levels of 4 g or less per 100 ml blood when started on the repletion assay which lasted four weeks.

Blumberg and Arnold (1947) found that quantitative differences could be calculated at any time after the first week. However, taking the values at the end of four weeks they reported that the iron of ferrous sulfate was four to five times as available as that of ferric orthophosphate; which, stated in the inverse, means that the iron of the orthophosphate was only 20 to 25 percent as available as that of ferrous sulfate.

Thus, within a few years after the initiation of the iron enrichment program, an appreciable amount of information was available on the biological efficacy of the compounds being used. In spite of the poor performance of the phosphate salts in the rat assays they have not been replaced in certain applications because those of greater bioavailability were reputed to have detrimental effects on the food products. Aside from a study of iron absorption in human subjects from breads containing the four most used iron compounds (Steinkamp *et al.*, 1955) little attention has been paid to this problem until very recent years.

B. RECENT DEVELOPMENTS

During the late 1960s there were expressions of increased concern over the evidence of unacceptable degrees of iron deficiency in segments of the U.S. population, and proposals were made that the levels of iron be increased in enriched flour, bread and related cereal products. In 1970, the joint Nutrition Committee of the American Baker's Association and the Millers' National Federation organized an *ad hoc* committee to review all information on present practices and problems of iron enrichment of flour-based foods and, particularly, to assess the impact of the proposed increased levels of iron enrichment. The findings of the Iron Committee have been published in a booklet

entitled, "Report of the *Ad Hoc* Committee on Iron Enrichment of Wheat Flour and Baked Foods"[4] (Bass, 1972).

The booklet contains valuable information, much of it in tabular form, on the major manufacturers of iron compounds for food use, estimates of productive capacity, chemical and physical specifications for the different compounds, and estimates of the relative cost per pound of iron in each form. Of particular interest is the review of the virtues and disadvantages of the various compounds because no one of them has all of the required characteristics. Ferrous sulfate, fully accepted as possessing the most bioavailable iron and produced in large quantity at relatively low cost, is the compound of choice for bread and other baked goods where long shelf life is not a factor. It is too reactive to be used in flour or flour-containing mixtures where long shelf life is a factor. It is reputed to be unacceptable in crackers and to affect adversely the color of pasta products.

Reduced iron, because of its chemical inertness and low cost, has been an important source of iron in flour and other products which may face extended storage. However, its high density compared with flour makes it difficult to maintain stable blends of uniform iron content. Its dark color is reported to be objectionable in some products, and the smaller the particle size, important for its absorption from the gastro-intestinal tract, the greater its darkening effect. In particular, manufacturers of pasta products find reduced iron objectionable. Ferric orthophosphate and sodium iron pyrophosphate because of their light color and chemical inertness have succeeded, by default, to those applications for which the other two were not suited, even though both phosphates are relatively costly sources of iron.

An idea of the extent to which each of these compounds was used during 1970 in the enrichment of food groups derived from cereal grains may be obtained from the figures in Table 2, which was prepared from the data in the publication of Friend (1972). Many of our consultants seriously question the validity of the figures in the category "other." They suggest that this category arises from inaccuracies in responding to, or in the interpretation of, questionnaires on which such summaries are prepared. In particular, they do not believe that 28 percent of the iron used at the bakery for the enrichment of bread and other baked goods could be a different compound, and that this amount of iron should be divided in some proportion and added to the four well-identified

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[4] A limited number of the booklets is available from Millers' National Federation, 14 East Jackson Boulevard, Chicago, Illinois 60604.

TABLE 2

DISTRIBUTION OF IRON FROM SPECIFIED IRON COMPOUNDS

ADDED TO GRAIN PRODUCTS, 1970

Iron Compounds	Total bulk and wafer 1	Bulk			Enrichment wafers for bread and bakery products 5
		Commercial flour or bread 2	Macaroni, rice, corn-meals and hominy grits 3	Cereals, ready-to-eat and hot 4	
	Percent	Percent	Percent	Percent	Percent
1 - Reduced iron	31	64	16	[1]30-40	[1]0-5
2 - Ferrous sulfate	20	[1]10-20	3	[1]0-5	47
3 - Ferric orthophosphate	18	[1]1-5	37	[1]20-30	15
4 - Sodium iron pyrophosphate	16	- - -	25	[1]30-40	[1]5-10
5 - Ferric ammonium citrate	[1]0-5	- - -	- - -	[1]0-5	- - -
6 - Other	[1]10-15	[1]10-20	19	[1]0-5	28
Total	100	100	100	100	100

[1] Exact percentages withheld to avoid disclosing figures for individual companies.

Prepared from Friend (1972).

compounds. Except for these reservations, the data in Table 2 are of interest for the present discussion.

The figures in columns 2-5 of the table reflect the preferences of the manufacturers of the different foods for certain iron compounds in the enrichment of their products: reduced iron in flour enriched at the mill and bread baked from such flour; the preponderant use of the iron phosphate salts in macaroni, rice, and similar products and in breakfast cereals; the substantial amount of compounds, other than ferrous sulfate, in the enrichment of baked goods at the bakery level. Undoubtedly, it would be equally accurate to say that these figures reflect the avoidance of specific iron compounds in certain foods for the reasons which have been alluded to earlier.

It may be noted that in 1970 the four iron compounds comprised at least 85 percent of the total, of which reduced iron contributed 31, ferrous sulfate 20, ferric orthophosphate 18, and sodium iron pyrophosphate 16. One of our consultants, fully conversant with the use of iron compounds in food fortification, notes that, not only has there been an appreciable increase since 1970 in the total amount of iron used for enrichment, but also there has been a change in the use of different iron compounds. He offers the following estimates of the 1973 percentage use in food enrichment: reduced iron 40-55; ferrous sulfate 25-30; the two iron phosphates 15-25; and other compounds 1-5. Such figures would suggest an increase in the use of reduced iron and ferrous sulfate at the expense of the iron phosphates. This may be regarded as indicating that it is now being accepted that the phosphate salts are poor sources of bioavailable iron.

Recently, at least two attempts have been made to render ferrous sulfate less reactive chemically while preserving its good absorption from the gastrointestinal tract. The new products are variously referred to as ferrous sulfate coated (encapsulated, stabilized), suggestive of at least one method by which it was hoped the desired result would be obtained. Preliminary and very limited information is available on each product. Jackel and Belshaw (1971), in testing the Balchem Corporation product, reported that storage studies showed no untoward changes in baking mixes and family flour during a six to eight months period. Bell *et al.* (1972) discussed the performance of two new preparations of stabilized ferrous sulfate (Mallinckrodt Chemical Works) in accelerated storage tests in flour, indicating marked improvement. Samples of both types of such preparations have been found to be equivalent to regular ferrous sulfate in bioavailability assays in rats (Fritz, 1973). It seems likely that a less reactive form of ferrous sulfate will become commercially available, but at a higher cost. However, if calculated

on the basis of per pound of bioavailable iron, such a special ferrous sulfate would be less expensive than the two phosphate salts presently used.

C. EVALUATION OF BIOAVAILABILITY

1. Animal Assays

Another example of the increased interest in recent years in the problem of the iron fortification of foods has been the use by several laboratories of the hemoglobin repletion test as a means of assessing the factors which influence the bioavailability of iron (Amine and Hegsted, 1971; Amine *et al.*, 1972; Fritz and Pla, 1972; Fritz *et al.*, 1970; Morris and Greene, 1972; Pla and Fritz, 1970; Ranhotra *et al.*, 1971). Not only have many iron compounds and iron-enriched foods been compared as to the bioavailability of their iron content but also efforts are being made to evolve an official bioassay method under the auspices of the Association of Official Analytical Chemists.[5]

The results of the first collaborative experiment among eight U.S. and Canadian laboratories, using anemic rats or chicks have been reported (Pla and Fritz, 1971). The samples compared in each laboratory were reagent grade ferrous sulfate, which served as the reference standard, ferric orthophosphate, sodium iron pyrophosphate, and reduced iron. The eight laboratories carried out 10 assays (three on rats, seven on chicks).

Although there were differences in the response of the two animal species to the two phosphate salts and to the reduced iron, the responses were in the same rank order in each; all the values on these compounds were therefore averaged to produce the pooled mean data. Assigning a biological value of 100 to the ferrous sulfate, the ferric orthophosphate was assigned a mean biological value of 12, sodium iron pyrophosphate, 13, reduced iron 46. Although each of these mean values has a large standard deviation there is no reason to doubt the evidence for the superior bioavailability of the iron of the ferrous sulfate, the

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[5] Mr. James C. Fritz is Supervisory Research Chemist in the laboratories of the Division of Nutrition, Food and Drug Administration, and also the A.O.A.C. Associate Referee who directs the collaborative experiments by which the details of an official method are worked out (See Appendix B).

intermediate value for the reduced iron, and the low values for the iron in the form of either phosphate salt. Generally similar results were obtained by Amine *et al.* (1972) using the same samples of the iron compounds discussed above in bioassays with both rats and chicks. It is obvious that these more recent assays confirm the results of those carried out during the 1940s (see p 39).

2. Human Assays

C. V. Moore and coworkers in the early 1950s (Steinkamp, Dubach, and Moore 1954, 1955) discussed the flour and bread enrichment program in terms very similar to those applied today. They stated that the four different preparations most commonly used (ferrous sulfate, reduced iron, ferric orthophosphate, and sodium iron pyrophosphate)

> "with the exception of ferrous sulfate, are certainly not the ones that one would expect to be best absorbed. Practical industrial problems have largely dictated their selection, in spite of observations indicating that solubility, ease of ionization, and the ferrous valence state are properties which lead to the greatest assimilation of iron" (Steinkamp *et al.*, 1955).

To investigate the bioavailability of the iron in these compounds they arranged to have each of them synthesized containing ^{59}Fe of high specific activity. It is to be noted that the methods of synthesis were devised in one laboratory (Merck and Co.) but applied in another (Abbott Laboratories). The protocols indicate that the sodium ferric pyrophosphate contained an excess of sodium pyrophosphate. Inasmuch as the double salt was used on the basis of its iron content the excess of the sodium salt was considered to be of no consequence. The reduced iron was characterized only as "finely powdered."

Each of the four radiolabeled iron compounds was baked into bread under commercial conditions, the formula being constant except for the source of iron. Because the ^{59}Fe salts were of high specific activity, unlabeled carrier salts were used to bring the level of added iron to 12 mg per pound of flour. The loaves were sliced by a mechanical slicer and four slices (approximately 100 g; 2 to 4 mg Fe were eaten with butter as breakfast by each subject after an overnight fast.

The subjects were 26 healthy male medical students and 6 healthy women. In addition, 3 hospital patients with hypochromic

anemia due to blood loss ate the bread containing ferrous sulfate, ferric orthophosphate, or sodium iron pyrophosphate, respectively. Although feces were collected and assayed for radioactivity, absorption was determined essentially on the ^{59}Fe which appeared in the circulating blood as newly synthesized hemoglobin. After several weeks one of the following control studies was done using the same subjects: (a) the same amount of ^{59}Fe compound as had been consumed in bread was given alone; (b) the same amount of the ^{59}Fe compound plus the same amount of unenriched bread; (c) the same amount of ^{59}Fe bread was eaten with a supplement of ascorbic acid.

The results of the experiment were summarized as follows. When bread, enriched with radioactive ferrous sulfate, with reduced iron, with ferric orthophosphate, or with sodium ferric pyrophosphate was fed to 32 healthy men and women, 28 of the subjects absorbed and utilized between 1 and 12 percent of the iron, regardless of the form of iron added. The other 4 subjects assimilated from 26 to 38 percent of the iron but there was reason to believe that their iron stores were suboptimal.

Ferrous sulfate baked into bread and free ferrous sulfate fed with unenriched bread were both absorbed to about the same extent. When ferrous sulfate was fed alone it was absorbed several fold better than from bread.

When radioactive reduced iron, ferric orthophosphate, or sodium ferric pyrophosphate was given as such together with unenriched bread, the level of absorption was comparable to that from the labeled bread.

When the bread, containing either of the four radioiron compounds, was fed with a supplement of ascorbic acid to six healthy subjects, iron absorption was increased two to three times.

When three iron-deficient hospital patients ate bread enriched with ^{59}Fe ferrous sulfate, ferric orthophosphate, or sodium ferric pyrophosphate, they absorbed 45 to 64 percent of the iron.

Although the authors expressed surprise at the lack of evidence of a difference in efficacy among the four forms of iron, they did comment on the low level of absorption by all the subjects, except the four with the suspected low iron stores. Greater assimilation could have been expected, because the test meal contained only 2-4 mg of iron. Later, two of the authors (Moore and Dubach, 1956) offered the following explanation of the above results: "Apparently the various iron preparations are changed in the baking process . . . so that all are about equally effective."

The above findings of Steinkamp *et al.* (1955) were favorably reviewed and generally accepted (Anonymous, 1955) and one may assume that the effect was to give a feeling of satisfaction with the bread enrichment program as it was being practiced commercially. In any event it was to be approximately 15 years before the efficacy of different forms of iron in enriched bread was fully reexamined in the United States with human subjects. In the meantime others (Höglund and Reizenstein, 1969; Vellar *et al.*, 1968) had reported evidence of differences in iron absorption from breads enriched with ferrous sulfate or with reduced iron; in particular the greater efficacy of the very finely divided reduced iron had been reported. The studies of Leichter and Joslyn (1967) indicated that the chemical events taking place during the fermentation and baking of bread were not such as to offer support for the speculation of Moore and Dubach (1956), that all forms of iron might lose their identity during baking and end in a common form.

A further important consideration was the realization that the sodium ferric pyrophosphate used by Steinkamp *et al.* (1955) was certainly different from the sodium iron pyrophosphate used commercially by the baking and cereal industries. The former was reported to be a green powder while the latter is white or off-white (Cook *et al.*, 1973). For these, and other reasons a collaborative experiment was planned involving the Division of Hematology, Department of Medicine, University of Washington School of Medicine, Seattle; the Division of Hematology, Department of Medicine, Washington University School of Medicine, St Louis; and the American Institute of Baking, Chicago, to study certain aspects of the absorption of radioactive iron supplements baked into dinner rolls and consumed by human volunteers (Cook *et al.*, 1973).

The relatively small amounts of the radiolabeled iron supplements were prepared by Abbott Laboratories under specifications agreed upon by representatives of major manufacturers of iron compounds for food enrichment and fortification. Ferrous sulfate (^{55}Fe and ^{59}Fe), ferric orthophosphate (^{59}Fe) and iron reduced by hydrogen (^{55}Fe) were synthesized essentially as described in the publication of Steinkamp *et al.* (1955). The sodium iron pyrophosphate (^{55}Fe) was prepared by a method furnished by Stauffer Chemical Company under a secrecy agreement with Abbott Laboratories. It was agreed that the iron content, particle size, and solubility were important criteria for suitability of the iron compounds for the experiment. The two preparations of ferrous sulfate were 100 percent soluble in water; the other three compounds were 100 percent soluble in 1.2M HCl. The reduced iron was milled to particles of 5-10 μ in size except for a few larger agglomerates, and hence was considered to be more finely divided than that commercially

available. The other labeled iron compounds were composed of equally small particles.

The rolls were prepared by the American Institute of Baking from a standard formula, the labeled iron compounds being added to the mixed drug ingredients using a portion of the formula water. After fermentation, punching and molding, the dough was subdivided into exactly equal portions to provide the required number of rolls per package. The dough pieces were proofed and baked on aluminum foil-lined sheets and after cooling were wrapped in the foil and the package placed in a plastic bag. These were then frozen and shipped in the frozen state to both Seattle and St. Louis. Measurements of the ^{59}Fe activity of the packages indicated a variation of less than ± 2 percent between packages containing the total dose of radioactivity for each subject.

Iron absorption tests were performed in healthy subjects in Seattle and St. Louis, the large majority of whom were females. The rolls containing the radioactive iron compounds were eaten between 7 and 9 a.m. after an overnight fast, and nothing further was taken by mouth for two hours (except when the rolls were part of a larger meal). Radioiron absorption was assessed from the activity found in the subject's blood two weeks after the test dose. Total blood volume was estimated, based on sex, height, and body weight, and it was assumed that the activity found in the blood represented 80 percent of total absorption.

Several separate studies were conducted as part of the whole experiment. The initial test concerned the bioavailability of the four iron compounds which had been baked into the rolls, the results of which are obviously of most immediate interest. Using a total of 24 subjects (22 ♀, 2 ♂), 3 groups of 8 each, comparison was made in each subject of the absorption from rolls tagged simultaneously with ^{55}Fe ferrous sulfate and one of the other three forms of iron labeled with ^{59}Fe. In one-half of these individual comparisons (4 in each group) the two iron supplements (1.5 mg iron each) were combined in the same 60 g roll and administered on 5 successive mornings. In the other half of the subjects two 30 g rolls, each containing 1.5 mg iron, either as labeled ferrous or as the labeled alternate iron supplement were administered together on 5 successive mornings. Two weeks later the ^{59}Fe and ^{55}Fe activities in the blood of the subjects were measured.

Immediately following these tests, a standard absorption test was performed on all of the 24 subjects. This consisted of administering for 5 mornings a reference dose of 3 mg ^{59}Fe as ferrous sulfate combined with ascorbic acid (2 moles ascorbic acid per mole of Fe) as a water solution. In blood samples obtained two weeks later, the

absorption of iron from this reference dose of ferrous ascorbate was determined from the increase in the ^{59}Fe activity over the previous level.

The results of the above study are summarized in Table 3 and illustrated in graphic form in Figure 3, from Cook *et al.* (1973).

In Table 3 the absorption of the labeled supplements is expressed, not only as percentage absorption, but also in relation to the absorption of the reference dose of ferrous ascorbate in each subject. This method of expression very largely compensates for subject-to-subject variation. The multiple daily doses help to reduce, but undoubtedly do not completely eliminate, the day-to-day variation in individual subjects. Because of the skewed distribution of iron absorption data, both the mean percentage absorption and the ratio between supplement and ferrous ascorbate absorption were calculated using the logarithmic scale and later retransformed as antilogarithms to recover original units.

The results show that the least available of the iron supplements was sodium iron pyrophosphate with a geometric mean absorption of 0.3 percent and a mean absorption ratio, relative to ferrous sulfate in the rolls, 0.05. Greater availability was observed for ferric orthophosphate as shown by the geometric mean absorption of 1.1 percent and a mean absorption ratio relative to ferrous sulfate of 0.31. Still greater availability relative to ferrous sulfate was observed for the supplement of iron reduced by hydrogen which had a mean absorption of 8.6 percent and a mean absorption ratio of 0.95. The individual absorption ratios are shown graphically in Figure 3.

These data may be summarized in the following way. Expressing the bioavailability of ferrous sulfate baked into rolls as 100 percent, that of the other supplements were: reduced iron 95 percent, ferric orthophosphate 31 percent, sodium iron pyrophosphate 5 percent. Of lesser import here, but of interest nevertheless, was the observed absorption of ferrous sulfate from the rolls in comparison with that from the reference dose. The mean absorption ratios calculated from the 3 separate groups were 0.33, 0.22, and 0.26 with an overall mean of 0.26. Therefore, the absorption of ferrous sulfate iron from the rolls was approximately one-fourth that from the ferrous sulfate solution containing ascorbic acid.

Cook and his associates (1973) carried out other tests as part of their overall study of the factors affecting the absorption of iron from supplements baked into dinner rolls. Investigations included: absorption as influenced by varying levels of iron supplementation; comparison of the absorption of biosynthetically labeled wheat iron with that

TABLE 3

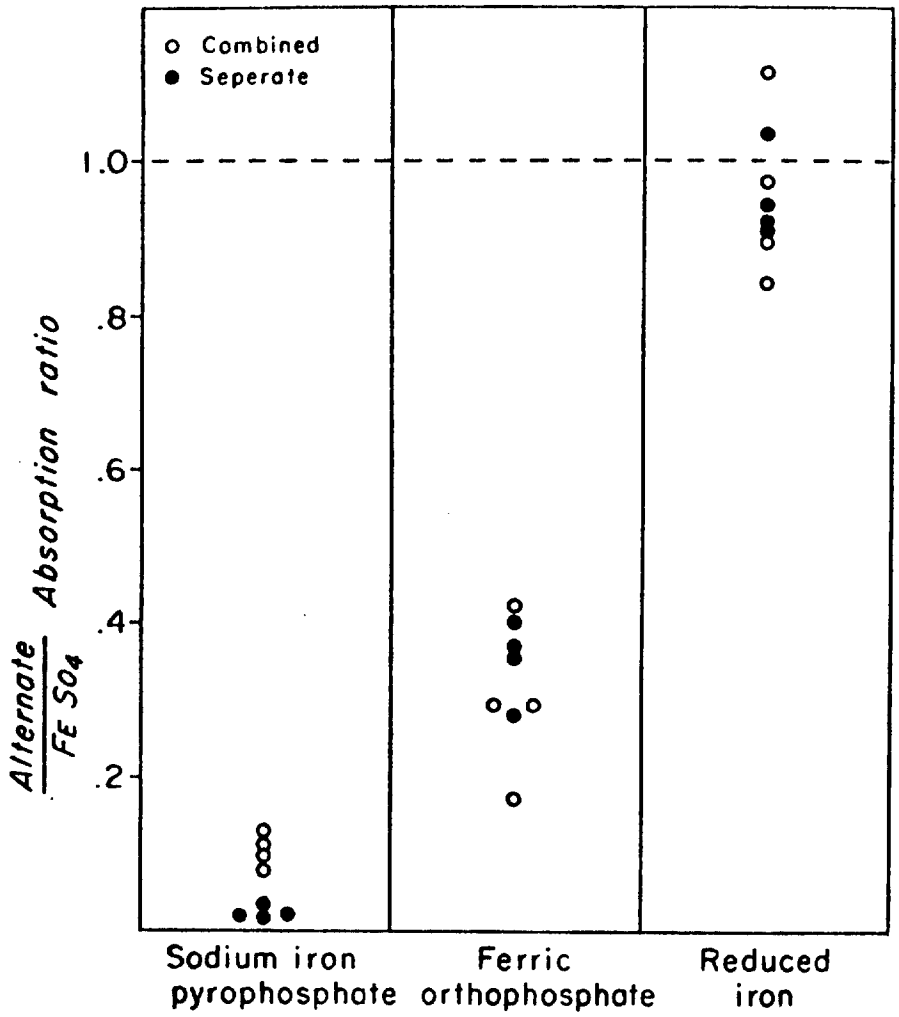
ABSORPTION OF VARIOUS RADIOIRON SUPPLEMENTS BAKED INTO ROLLS

Subject	Sex and age	Hct, %	Serum iron, μg/100 ml	Transferrin saturation, %	Administration	Iron absorption, %			Absorption ratios		
						A Ferrous sulfate	B Alternate	R Reference	A/R	B/R	B/A
A. Alternate, sodium iron pyrophosphate											
1 JW	F21	42	116	38	Combined	4.1	0.1	38.4	0.11	0.00	0.02
2 HH	F20	41	44	13	Combined	6.1	0.2	38.6	0.16	0.01	0.03
3 ZH	F19	43	94	22	Combined	16.0	0.3	40.5	0.40	0.01	0.02
4 JH	F26	43	88	32	Combined	24.6	0.5	90.8	0.27	0.01	0.02
5 DMc	F44	40	120	37	Separate	0.9	0.1	6.3	0.14	0.02	0.11
6 MM	F28	43	67	17	Separate	28.8	2.4	24.4	1.18	0.10	0.08
7 JB	F22	44	96	25	Separate	6.3	0.6	14.4	0.44	0.04	0.10
8 JN	F24	43	187	38	Separate	2.3	0.3	2.1	1.10	0.14	0.13
Mean[a]		42	102	28		6.6	0.3	20.0	0.33	0.02	0.05
B. Alternate, ferric orthophosphate											
1 KH	F22	39	172	53	Combined	10.9	4.0	88.3	0.12	0.05	0.37
2 MM	F19	43	103	25	Combined	2.9	0.8	18.5	0.16	0.04	0.28
3 SD	F29	42	143	40	Combined	1.5	0.6	7.1	0.21	0.08	0.40
4 CS	F21	41	92	31	Combined	11.5	4.1	40.1	0.29	0.10	0.36
5 AC	F39	43	100	24	Separate	2.1	0.6	4.4	0.48	0.14	0.29
6 EI	M19	50	146	39	Separate	3.3	1.4	18.8	0.18	0.07	0.42
7 GS	M29	47	100	26	Separate	3.1	0.9	10.4	0.30	0.09	0.29
8 BS	F28	39	204	39	Separate	2.4	0.4	13.3	0.18	0.03	0.17
Mean[a]		43	133	35		3.6	1.1	16.4	0.22	0.07	0.31
C. Alternate, reduced iron											
1 RR	F22	42	142	39	Combined	4.0	4.1	30.9	0.13	0.13	1.03
2 NM	F20	40	81	22	Combined	5.5	5.0	40.5	0.14	0.12	0.91
3 JB	F19	43	68	22	Combined	16.6	15.3	34.8	0.48	0.44	0.92
4 TN	F29	41	65	20	Combined	48.9	45.8	100.4	0.49	0.46	0.94
5 LI	F49	40	63	15	Separate	21.5	23.9	73.5	0.29	0.33	1.11
6 IR	F22	42	89	25	Separate	5.9	5.3	24.9	0.24	0.21	0.90
7 CS	F23	41	72	26	Separate	6.6	6.4	16.3	0.40	0.39	0.97
8 NF	F24	43	139	36	Separate	3.1	2.6	15.4	0.20	0.17	0.84
Mean[a]		42	90	26		9.1	8.6	34.5	0.26	0.25	0.95

[a] Geometric means have been calculated for iron absorption and absorption ratio values.

From Cook *et al.* (1973).

FIGURE 3



Absorption of iron by each subject from indicated alternate supplement relative to that from ferrous sulfate. The alternate supplement and ferrous sulfate were added either to the same 60 g roll (combined) or to separate 30 g rolls eaten at the same time (separate). From Cook *et al.* (1973).

from labeled supplemental ferrous sulfate; and absorption from supplemented rolls eaten as part of different complete meals. While interesting and important data were obtained from each study, no detailed discussion of them will be attempted because they are somewhat peripheral to the main concern of this report.

From the results presented by Cook *et al.* (1973), there can no longer be any doubt as to the poor bioavailability of phosphate salts of iron, first noted in human subjects by Moore *et al.* (1939) and repeatedly demonstrated in animal experiments both before and after that date. The most recent animal experiments (Pla and Fritz, 1971) would suggest that there is less difference in efficacy between ferric orthophosphate and sodium iron pyrophosphate than that found by Cook *et al.* (1973). Admittedly, the animal experiments in different laboratories gave widely divergent results and, certainly, the hemoglobin repletion test can be carried out with greater precision than was achieved in the first collaborative assay (Pla and Fritz, 1971). Also it might well be that another study in humans, similar to that of Cook *et al.* (1973) would yield somewhat different results. But an increase in assay precision, as regards the phosphate salts, most probably would be quite academic.

The relatively high bioavailability of reduced iron, essentially equivalent to ferrous sulfate, reported by Cook *et al.* (1973) is surprising. Animal experiments have given values in the 40-60 percent range (chicks somewhat better than rats). Cook and his associates have emphasized that they used a sample with particles ranging in size from 5 μ to 10 μ (except for a few larger agglomerates) and that this was smaller than is commercially available. However, Höglund and Reizenstein (1969) report using a sample of reduced iron, 97 percent of which had particles about 5 μ in size, which they compared with a sample, 23 percent of which was 25 μ and 48 percent was larger than 30 μ in size. Both of these and also ferrous sulfate were added to flour (40 mg Fe/kg) and baked into bread. The absorption by human subjects of iron from the "fine grain" bread was 50 percent, from the "coarse grain" bread 16 percent of that absorbed from the ferrous sulfate bread. This difference between the findings of Cook *et al.* (1973) and Höglund and Reizenstein could be explained by the fact that the latter used powdered iron prepared by a different method (See Appendix A).

Similar results, indicating an effect of particle size, were obtained by Motzok *et al.* (1973) in a hemoglobin repletion test on rats. The reduced iron was separated by air elutriation into several samples; those of 7-10 μ, 14-19 μ and 27-40 μ were tested in comparison with ferrous sulfate. The results showed that the three samples had the

following relative biological values: 45, 22, and 12 with ferrous sulfate having the assigned value of 100. In a similar test using chicks the same samples gave relative values of 65, 49, and 41, respectively.

VI. IRON NUTRITION OF INFANTS AND CHILDREN

The human infant, like other newborn mammals, is born with a variable store of body iron and subsists during early life on an iron-poor diet. Although breast milk contains somewhat more iron than cow's milk, this difference is inconsequential because neither contains enough to maintain iron balance in the infant during the period that milk forms the sole, or main, item of the diet. The rate of physical growth is faster and the need for iron is greater during the first year of life than at any other time (Andelman and Sered, 1966). Consequently, the body store of iron with which an infant is born assumes great importance. This initial iron endowment ultimately proves to be inadequate and dietary stores of iron must be provided if iron deficiency is to be avoided. Supplemental foods do not always provide sufficient iron and iron-deficiency anemia is a prevalent nutritional disorder among infants and children in the United States (Foman, 1970).

Without attempting a detailed review of the pediatric literature, several published papers have been selected for brief discussion because of the light which they throw on the need for iron supplementation and the recommendations as to how this may be achieved in the feeding of infants. At the outset it should be acknowledged that the findings in this field are not very helpful in assessing quantitatively the relative bio-availability of the iron sources which have been used.

Elvehjem *et al.* (1933) were among the first to determine the average level of hemoglobin in a large population of normal infants from birth to five years of age. All the children were from white middle-class families, fed according to accepted pediatric practice but without specific iron supplementation. Hemoglobin values from premature infants, twins, and children definitely anemic were excluded from the tabulation. Table 4, covering the first two years of life, was prepared from the data of Elvehjem and his associates (1933).

It is of interest to note the high levels of blood hemoglobin at birth and its precipitous drop during the early weeks of life; also the many weeks during the first two years that average hemoglobin falls between 11 and 12 g per 100 ml of blood. Note should also be taken of the highest and lowest values in each group indicating the range of values hidden in the averages.

The above study was preliminary to a second in which the effect on hemoglobin levels of the addition of iron and copper to the diet of

TABLE 4

HEMOGLOBIN CONTENT OF BLOOD OF NORMAL INFANTS
IN MADISON, WISCONSIN
DURING THE FIRST TWO YEARS OF LIFE

Age in Weeks	Number of Determinations	Gm. Hemoglobin per 100 ml Blood		
		Lowest	Highest	Average
0-2	21	18.8	27.0	22.2
2-4	27	13.2	21.9	17.9
4-8	89	8.3	19.4	13.4
8-12	118	8.8	17.6	11.7
12-16	126	9.1	15.5	11.9
16-20	135	8.3	15.5	12.0
20-24	141	8.2	15.1	12.2
24-28	116	8.5	16.0	11.8
28-32	118	8.0	15.2	11.6
32-36	118	8.5	15.7	11.8
36-40	110	8.8	14.9	11.5
40-44	91	8.0	14.0	11.4
44-48	84	8.0	14.9	11.5
48-52	77	8.4	15.1	11.6
52-56	82	9.4	14.7	11.5
56-60	63	8.3	14.2	11.6
60-64	41	8.0	13.7	11.1
64-68	45	9.0	14.1	11.6
68-72	38	9.4	13.9	11.0
72-76	39	8.8	15.9	11.4
76-80	32	8.7	16.1	11.4
80-88	50	8.4	13.7	11.8
88-96	45	10.0	13.6	11.9
96-104	51	8.9	14.4	11.9
104-112	33	9.4	14.0	11.9

Prepared from the data of Elvehjem *et al.* (1933).

infants was investigated (Elvehjem *et al.*, 1935). Again the subjects were healthy infants brought to one of the health centers of the Madison (Wisconsin) Public Health Nursing Association. An effort was made to select younger infants coming to the clinic so that the administration of the iron and copper could be started as early as possible.

The combination of iron in the form of ferric pyrophosphate and copper as copper sulfate was prepared either as a solution or as tablets, each preparation containing 25 mg Fe to 1 mg Cu. The authors state that the ferric pyrophosphate and the copper sulfate were soluble in distilled water containing 5 percent alcohol. The solution or a tablet was added to the infant's formula, orange juice, or a glass of milk to provide daily the above amounts of the two metals. Figure 4 illustrates the average hemoglobin curve of the 47 infants that received the combined supplement in comparison with the average curve for the untreated infants. It is evident that the iron and copper had a distinct effect in increasing the average level of hemoglobin during the period of 8 to 56 weeks of age.

The authors acknowledged that when cereal or other supplemental food was added to the infant's diet it was less likely to be deficient in copper than a diet of milk alone. However, no study was made in which iron alone was compared with the combination of iron and copper, by which the value of might have been assessed. Because subsequent investigations have not established a need for copper in the prevention of anemia in infants fed supplemental foods at an appropriate age, one must assume that benefit from the combination used by Elvehjem *et al.* (1935) was due, mainly if not entirely, to the iron. Obviously, no judgment can be formed from these results concerning the relative bioavailability of the iron in ferric pyrophosphate beyond noting that the high daily intake supplied enough to increase hemoglobin formation.

The first infant cereal, fortified with certain vitamins and high in iron, to be made available commercially was that[6] described by Tisdall *et al.* (1930). The iron content of 25-30 mg per 100 g was derived largely from iron-rich ingredients (bone meal, wheat germ, and alfalfa). Stearns and Stinger (1937) studied the retention of iron in 13 infants varying from 7 to 54 weeks of age throughout a total of 98 three-day balance periods. The infants were given a basal diet of evaporated cow's milk, carbohydrate, cod liver oil concentrate, and orange juice. On the basal diet alone the infants lost an average of 0.05 mg iron daily Neither egg yolk nor pureed spinach, in the amounts given, increased

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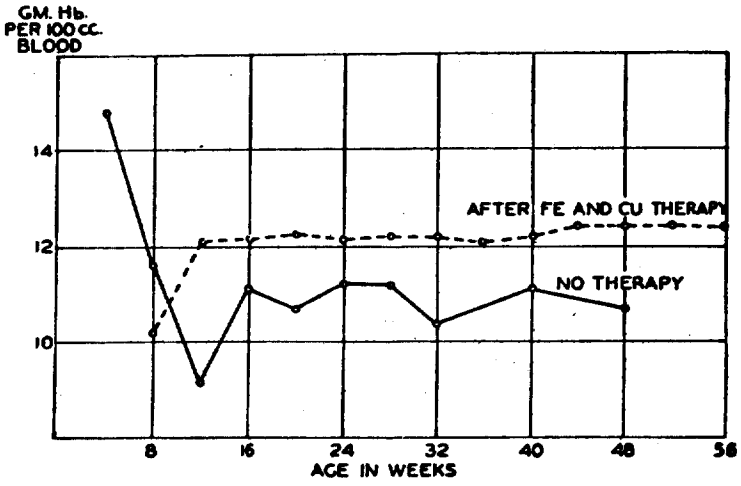
[6] Pablum®

FIGURE 4



Average hemoglobin level of 47 infants receiving 25 mg Fe and 1 mg Cu daily compared with that of infants receiving no supplement. From Elvehjem *et al.* (1935).

iron retention, but the retentions were definitely increased when infants were given the special iron-rich cereal or a solution of ferric ammonium citrate. Based on the iron retentions following the ingestion of varying amounts of iron in the supplemental foods (including the special cereal) or as the soluble iron salt, it was estimated that an intake of approximately 0.5 mg per kg body weight daily was necessary to secure some retention. An intake of 1 to 1.5 mg per kg daily permitted ample retention. No difference was noted in the efficacy of the iron in the cereal in comparison with that from the ferric ammonium citrate.

A later investigation in the same laboratory (Niccum *et al.*, 1953) was undertaken to determine the amount and the form of iron best suited for the prevention of hypochromic anemia in infants. Various amounts of iron had been used or recommended by other investigators as a daily dose and in the meantime evidence for the superiority of ferrous iron over ferric iron had accumulated. In earlier work this laboratory had used a solution of ferric ammonium citrate and this use had been continued in the intervening years. The usual management of the infants in the metabolism ward was to give 5 mg of iron daily from this source beginning at 3 months of age and to increase this to 10 mg daily after 6 months of age. The iron was always added to the formula.

In order to determine the efficacy of a ferrous form of iron, a commercial solution of ferrous sulfate was administered to 18 full-term infants in the metabolism ward for 6-to-12-month periods. The dosage given was the same as for the ferric ammonium citrate. The hemoglobin levels recorded for these subjects were compared with the records of 115 full-term infants that had received ferric ammonium citrate and had been in the metabolism ward for 6-to-12-month periods in earlier years. In addition to the infants in the ward, 19 full-term infants were observed in a well-baby clinic who also received the ferrous sulfate. Several premature or low birth-weight babies were also available for the comparison between the two iron salts.

Niccum *et al.* (1953) concluded that the ferrous iron, in the same dosage, supported significantly higher hemoglobin values in infants than the ferric iron. They also were convinced that the doses which they administered, small by comparison with those given by other investigators, were sufficient to maintain hemoglobin values at a constant level throughout the second half of infancy in all full-term infants. Even in those infants who had severe infections, marked anemia did not develop and hemoglobin values were maintained above 10 g per 100 ml.

An obvious comment that one may make about these findings is that there is little reason to doubt the greater efficacy of the ferrous sulfate but the difference would have to be accepted as qualitative rather than quantitative.

The absorption of iron from foods by infants and children was studied by Schulz and Smith (1958). The subjects, ranging in age from 3 months to 15 years, were all in apparent good health; a few were iron-deficient. By injecting ^{59}Fe into a milking cow and into laying hens, the endogenous iron of milk, eggs, and chicken liver was labeled. Also included in the study was a brand of commercial infant cereals, enriched with radiolabeled sodium iron pyrophosphate at a level of 50 mg iron per 100 g. The absorption of iron was measured by the balance method; the difference between the radioactivity ingested and that appearing in the stools was taken to represent the amount absorbed.

Schulz and Smith (1958) reported that normal children absorbed an average of about 10 percent of the naturally occurring iron in milk, eggs, and chicken liver, and of the iron supplement added to commercially prepared infant cereals. Iron deficient children absorbed two to three times the amount of food iron absorbed by normal children. The mean absorption of iron from eggs was higher in younger children than in older children. Normal children absorbed more iron from milk than normal adult males by about 5 to 1.

These results are difficult to interpret; particularly because the iron intake from the fortified cereals, containing sodium iron pyrophosphate, was several times that from milk, and more than twice that from labeled eggs. The similar percentage absorption from all the supplements suggests that the method of measuring absorption may not have been adequate.

Several subsequent investigations, carefully carried out, have demonstrated the value of iron added to the diet of infants, full-term and premature, from birth the one year of age in maintaining hemoglobin at acceptable levels. In three of these investigations (Andelman and Sered, 1966; Gorten and Cross, 1964; Marsh *et al.*, 1959) a comparison was made of a commercial infant formula containing 12.5 mg iron per quart (as ferrous sulfate) with the formula without added iron. Another (Moe, 1963) studied the utility of ferrous saccharate added to an infant cereal which was fed twice a day beginning at about $3\frac{1}{2}$ months of age. These studies, like those discussed earlier, attest to the need of the infant for exogenous sources of iron beyond that provided by milk, and as exemplified by the infants who received the prepared formula or cereal without added iron, the early feeding of ordinary foods regarded as

sources of iron (eggs, strained meats, fruits, vegetables) were inadequate to prevent undesirable degrees of iron deficiency in many of the infants in those groups.

The Committee on Nutrition, American Academy of Pediatrics, (1969) reviewed the problem of iron balance and requirements during infancy and has recommended that the diet of the normal term infant provide 1.0 mg of iron per kilogram of body weight per day by 3 months of age to a maximum intake of 15 mg/day. A somewhat greater iron allowance (2 mg/kg/day) is recommended for low birth weight (smaller iron endowment) infants beginning at 2 months of age to a maximum intake of 15 mg/day. These requirements can be met with certainty only by the inclusion in the diet of foods which have been fortified with iron, such as infant cereals or prepared formulas. Determined by overall use, the most important source of iron for infants is the cereals which in the main are enriched by the addition of reduced iron or sodium iron pyrophosphate to levels of between 8.6 and 22 mg iron per dry ounce.

Aside from the publication of Niccum *et al.* (1953), none of those reviewed above has dealt with the problem of possible differences in efficacy among the forms of iron that were used. Most of the investigators were concerned with the practical problem of how best to protect infants and children from iron deficiency, and in general they have used those commercial sources which were easily available. This situation is recognized in the statement of the Committee on Nutrition, American Academy of Pediatrics (1969) where it is recommended that efforts should be made to determine more precisely the best form of iron to be used in the supplementation of foods for infants and children. There is good evidence that the latter absorb more iron than adults from whatever source is provided, a reflection, undoubtedly, of low iron stores, rapid growth and expanding hemoglobin mass. There is little reason to doubt that, when quantitatively measured, infants and children will show the same discrimination in bioavailability among the different iron compounds as is shown by adults and experimental animals. To the present this has not been demonstrated adequately.

Finally, reference may be made to recent findings (Hodson, 1970; Theuer *et al.*, 1971; 1973) which indicate that relatively insoluble iron salts when added to liquid nutritional preparations (weight control dietaries, infant formulas) gradually become soluble. This increasing solubility allows the salt to ionize and the Fe atom to be reduced and/or to react with other anions. Hodson (1970), working with a liquid dietary containing added vitamins (including ascorbic acid) and ferric orthophosphate, found by chemical analysis that there was, with time, an increasing proportion of the iron present in the ferrous valence.

Theuer *et al.* (1971, 1973) assessed by the hemoglobin repletion method the bioavailability of various iron salts added to infant formulas which were sampled at different times during the process of manufacture. In particular, ferric pyrophosphate and sodium iron pyrophosphate were compared with ferrous sulfate when, (a) added directly to the anemia-producing basal diet, (b) after being carefully blended into different portions of the same batch of formula, and (c) after these same portions had been subjected to the final heat sterilization process. The assay results indicated that the bioavailability of the insoluble iron salts in the samples of formula which had been heat sterlized was significantly better than those which had not been heated. No attempt was made to determine if extended storage had any additional effect on the bioavailability of the iron in the sterilized preparations.

VII. RESUMÉ

It is apparent that in the United States the iron enrichment of foods is accomplished almost entirely through the use of four compounds: ferrous sulfate, reduced iron (so-called), ferric orthophosphate, and sodium iron pyrophosphate. In the regulation establishing the original flour and bread enrichment program (Federal Register, 1941), the iron to be added was described only as "harmless and assimilable." Considering the assay values which have been determined for either ferric orthophosphate or sodium iron pyrophosphate, it would be difficult to interpret "assimilable" in a manner which would include either compound. The former has a value of 25 to 30 percent that of ferrous sulfate (Blumberg and Arnold, 1947; Cook *et al.*, 1973); the latter a value of 5 to 10 percent (Cook *et al.*, 1973; Pla and Fritz, 1971). It seems obvious that, from the point of view of the consumer, the addition of ferric orthophosphate to a cereal food is a very minor nutritional benefit, and the addition of sodium iron pyrophosphate is essentially futile. Therefore, ir would seem wise to examine each present use of these salts in the hope that an iron compound of greater bioavailability might be substituted.

Some of our consultants were firmly of the opinion that fullest use was not being made of ferrous sulfate at the present time; particularly so at the bakery level. The Iron Committee (Bass, 1972) has stated that "the use of ferrous sulfate at the bakery level appears to create no problems," but, for reasons of convenience or reluctance to change, other forms of iron are still used. The belief was expressed that if all enrichment of bread and other baked goods were carried out at the bakery this would promote a greater use of ferrous sulfate. As noted in Table 2 when enrichment is done at the flour mill the iron added is preponderantly reduced iron.

A development of great potential for extending the use of ferrous sulfate in the enrichment program would be the commercial availability of a stabilized form of this salt. The objective is to treat the compound in some manner so that its untoward effects on flour or other food during storage is inhibited while its good absorption from the digestive tract is retained. The problem is under active investigation and there is reason to believe that it will be solved in some degree.

The most important iron source in the enrichment of foods is, by present estimates of use, reduced iron. In an addendum to this report (Appendix A), Patrick has provided valuable and much-needed

information on the several iron powders which are collectively referred to as "reduced iron" in the food industry. He describes the process by which each is manufactured and suggests how this may influence the presence of impurities, particle size distribution, and biological activity. It is obvious that the extent to which the particles of iron dissolve in the acid of the stomach must be a very important determinant of bioavailability, but it is indicated that not only the size of the particle but also its shape, surface area and density influence the speed with which it dissolves. The differences in the latter properties are suggested in the illustrations.

The possible effect of the method of manufacture on the biological properties of iron powders would suggest that distinctive names should be applied to each. The Food Chemicals Codex (1972) contains monographs on "Iron, Electrolytic" and "Iron, Reduced" but none on iron prepared by the carbonyl method. This could indicate that no carbonyl iron powder is used in the enrichment of foods in this country, but because of the present imprecise designation, "reduced iron," this assumption might be wrong. This situation should be clarified.

The excellent absorption of iron reduced by hydrogen observed by Cook *et al.* (1973) in human subjects was ascribed, in large part, to the very small particles achieved in milling the small radiolabeled sample. These authors reported that more than 95 percent of the particles ranged in size from 5 μ to 10 μ, which they acknowledged was smaller than is commercially available. Even though it would be unrealistic economically, and, according to Patrick, perhaps hazardous to attempt the commercial production of such extremely fine iron powders, efforts in this direction should be considered. Certainly, in the use of iron powders for food enrichment, average particle size distribution must be an important specification. Cook *et al.* (1973) have listed the size distribution in the better commercial products.

In Appendix B certain comments and suggestions have been made in the hope of expediting the development of an official assay method for assessing the bioavailability of the iron in different sources. There is urgent need for an official well-designed assay. Without it there is little hope that the widely divergent values, that have been reported on the same products, can be resolved, or that further progress can be made in solving the remaining problems of the enrichment program.

VIII. BIBLIOGRAPHY

Amine, E.K., and D.M. Hegsted. 1971. Effect of diet on iron absorption in iron-deficient rats. J. Nutr. 101: 927-935.

Amine, E.K., R. Neff, and D.M. Hegsted. 1972. Biological estimation of available iron using chicks or rats. J. Agr. Food Chem. 20: 246-251.

Andelman, M.B., and B.R. Sered. 1966. Utilization of dietary iron by term infants. Amer. J. Dis. Child. 111: 45-55.

Anonymous. 1955. Utilization of iron compounds in enriched bread. Nutr. Rev. 13: 165-166.

Austoni, M.E., and D.M. Greenberg. 1940. Studies in iron metabolism with the aid of its artificial radioactive isotope. The absorption, excretion, and distribution of iron in the rat on normal and iron-deficient diets. J. Biol. Chem. 134: 27-41.

Bannerman, R.M. 1965. Quantitative aspects of hemoglobin-iron absorption. J. Lab. Clin. Med. 65: 944-950.

Bass, E.J., Chairman. 1972. Report of the *ad hoc* committee on iron enrichment of wheat flour and baked foods. American Bakers Association, Washington, D.C., and Millers' National Federation, Chicago, Ill. 31 pp.

Bell, A.D., M.A. Currie, R.S. Schreiber, and D.S. Titus. 1972. Page 99 *in* 57th annual meeting program, American Association of Cereal Chemists, Miami Beach, Fla., October 29-November 2, 1972. (Abstract 89)

Bing, F.C. 1972. Assaying the availability of iron. J. Amer. Diet. Ass. 60: 114-122.

Blaud, P. 1832. Sur les maladies chloratiques, et sur un mode de traitement spécifique dans ces affections, Rev. Médicale 1: 337-367.

Bliss, C.I., and C. White. 1967. Statistical methods in biological assay of the vitamins. Pages 21-137 *in* P. Gyorgy and W.N. Pearson, eds. The vitamins, vol. VI, 2nd ed., Academic Press, New York, N.Y.

Blumberg, H., and A. Arnold. 1947. The comparative biological availabilities of ferrous sulfate iron and ferric orthophosphate iron in enriched bread. J. Nutr. 34: 373-387.

Blumberg, H., D.H. Shelling, and D.A. Jackson. 1938. The production of manganese rickets in rats. J. Nutr. 16: 317-324.

Bothwell, T.H., and C.A. Finch. 1962. Iron metabolism. Little, Brown and Company, Boston, Mass. 440 pp.

Bothwell, T.H., G. Pirzio-Biroli, and C.A. Finch. 1958. Iron absorption. I. Factors influencing absorption. J. Lab. Clin. Med. 51: 24-36.

Branion, H.D., B.L. Guyatt, and H.D. Kay. 1931. Beryllium rickets. J. Biol. Chem. 92: xi.

Brise, H. 1962. Influence of meals on iron absorption in oral iron therapy. Acta Med. Scand. 171 (Suppl. 376): 39-45.

Brise, H., and L. Hallberg. 1962a. A method for comparative studies on iron absorption in man using two radioiron isotopes. Acta Med. Scand. 171 (Suppl. 376): 7-22.

Brise, H., and L. Hallberg. 1962b. Absorbability of different iron compounds. Acta Med. Scand. 171 (Suppl. 376): 23-37.

Brown, E.B., Y.F. Hwang, S. Nicol, and J. Ternberg. 1968. Absorption of radioiron-labeled hemoglobin by dogs. J. Lab. Clin. Med. 72: 58-64.

Callender, S.T., B.J. Mallett, and M.D. Smith. 1957. Absorption of haemoglobin iron. Brit. J. Haematol. 3: 186-192.

Chappelle, E., B.W. Gabrio, A.R. Stevens, Jr., and C.A. Finch. 1955. Regulation of body iron content through excretion in the mouse. Amer. J. Physiol. 182: 390-392.

Committee on Nutrition, American Academy of Pediatrics. 1969. Iron balance and requirements in infancy. Pediatrics 43: 134-142.

Conrad, M.E. 1970. Factors affecting iron absorption. Pages 87-120 *in* L. Hallberg, H.G. Harwerth, and A. Vannotti, eds. Iron deficiency. Pathogenesis, clinical aspects, therapy. Academic Press, New York, N.Y.

Conrad, M.E., B.I. Benjamin, H.L. Williams, and A.L. Foy. 1967. Human absorption of hemoglobin-iron. Gastroenterology 53: 5-10.

Conrad, M.E., S. Cortell, H.L. Williams, and A.L. Foy. 1966a. Polymerization and intraluminal factors in the absorption of hemoglobin-iron. J. Lab. Clin. Med. 68: 659-668.

Conrad, M.E., L.R. Weintraub, D.A. Sears, and W.H. Crosby. 1966b. Absorption of hemoglobin iron. Amer. J. Physiol. 211: 1123-1130.

Cook, J.D., M. Layrisse, C. Martinez-Torres, R. Walker, E. Monsen, and C.A. Finch. 1972. Food iron absorption measured by an extrinsic tag. J. Clin. Invest. 51: 805-815.

Cook, J.D., V. Minnich, C.V. Moore, A. Rasmussen, W.B. Bradley, and C.A. Finch. 1973. Absorption of fortification iron in bread. Amer. J. Clin. Nutr. 26: 861-872.

Council on Foods and Nutrition. [American Medical Association] 1941. Nutritionally improved or enriched flour and bread. J. Amer. Med. Ass. 116: 2849-2853.

Cox, G.J., M.L. Dodds, H.B. Wigman, and F.J. Murphy. 1931. The effects of high doses of aluminum and iron on phosphorus metabolism. J. Biol. Chem. 92: xi.

Day, H.G., and H.J. Stein, 1938. The effect upon hematopoiesis of variations in the dietary levels of calcium, phosphorus, iron and vitamin D. J. Nutr. 16: 525-540.

Deobald, H.J., and C.A. Elvehjem. 1935. The effect of feeding high amounts of soluble iron and aluminum salts. Amer. J. Physiol. 111: 118-123.

Diamond, L.K. 1970. Pages 17-18 *in* Iron nutrition in infancy, report of the Sixty-second Ross Conference on Pediatric Research, Ross Laboratories, Columbus, Ohio.

Elvehjem, C.A. 1932. The relative value of inorganic and organic iron in hemoglobin formation. J. Amer. Med. Ass. 98: 1047-1050.

Elvehjem, C.A., W.H. Peterson, D.R. Mendenhall. 1933. Hemoglobin content of the blood of infants. Amer. J. Dis. Child. 46: 105-112.

Elvehjem, C.A., A. Siemers, and D.R. Mendenhall. 1935. Effect of iron and copper therapy on hemoglobin content of the blood of infants. Amer. J. Dis. Child. 50: 28-35.

Federal Register. 6: 2574, May 27, 1941.

Federal Register 36: 23074, December 3, 1971.

Fomon, S.J. 1970. Prevention of iron-deficiency anemia in infants and children of preschool age. Health Services and Mental Health Administration. U.S. Dept. of Health, Education, and Welfare. PHS 2085, Government Printing Office, Washington, D.C. 19 pp.

Food Chemicals Codex, 1972. 2nd ed. National Academy of Sciences, Washington, D.C. 1039 pp.

Fowler, W.M., and A.P. Barer. 1939. The treatment of iron deficiency anemias. J. Amer. Med. Ass. 112: 110-114.

Freeman, S., and M.W. Burrill. 1945. Comparative effectiveness of various iron compounds in promoting iron retention and hemoglobin regeneration by anemic rats. J. Nutr. 30: 293-300.

Friend, B. 1972. Enrichment and fortification of foods, 1966-70. Pages 29-33 *in* National food situation, NFS-142, Economic Research Service, U.S. Department of Agriculture, Washington, D.C.

Fritz, J.C. 1973. Personal communication; discussion of stabilized ferrous sulfate.

Fritz, J.C., and G.W. Pla. 1972. Vitamins and other nutrients. Application of the animal hemoglobin repletion test to measurement of iron availability in foods. J. Ass. Offic. Anal. Chem. 55: 1128-1132.

Fritz, J.C., G.W. Pla., T. Roberts, J.W. Boehne, and E.L. Hove. 1970. Biological availability in animals of iron from common dietary sources. J. Agr. Food Chem. 18: 647-651.

Gorten, M.K., and E.R. Cross. 1964. Iron metabolism in premature infants. II. Prevention of iron deficiency. J. Pediatr. 64: 509-520.

Granick, S. 1954. Iron metabolism. Bull. N.Y. Acad. Med. 30: 81-105.

Hahn, P.F. 1937. The metabolism of iron. Medicine 16: 249-266.

Hahn, P.F., W.F. Bale, E.O. Lawrence, and G.H. Whipple. 1939. Radioactive iron and its metabolism in anemia; its absorption, transportation and utilization. J. Exp. Med. 69: 739-753.

Hahn, P.F., W.F. Bale, J.F. Ross, W.M. Balfour, and G.H. Whipple. 1943. Radioactive iron absorption by gastro-intestinal tract; influence of anemia, anoxia, and antecedent feeding distribution in growing dogs. J. Exp. Med. 78: 169-188.

Hahn, P.F., E. Jones, R.C. Lowe, G.R. Meneely, and W. Peacock. 1945. The relative absorption and utilization of ferrous and ferric iron in anemia as determined with the radioactive isotope. Amer. J. Physiol. 143: 191-197.

Hart, E.B., H. Steenbock, J. Waddell, and C.A. Elvehjem. 1928. Iron in nutrition. VIII. Copper as a supplement to iron for hemoglobin building in the rat. J. Biol. Chem. 77: 797-812.

Hegsted, D.M., C.A. Finch, and T.D. Kinney. 1949. The influence of diet on iron absorption. II. The interrelation of iron and phosphorus. J. Exp. Med. 90: 147-156.

Heinrich, H.C. 1970. Intestinal iron absorption in man -- methods of measurement, dose relationship, diagnostic and therapeutic applications. Pages 213-296 *in* L. Hallberg, H.G. Harwerth and A. Vannotti, eds. Iron deficiency: pathogenesis, clinical aspects, therapy. Academic Press, New York, N.Y.

Henley, E.D., W.N. Christenson, W.J. Grace, and H.G. Wolfe. 1956. Absorption of iron from the gastrointestinal tract; a comparative study of the oral iron tolerance test in human beings using stable and radioactive iron. Amer. J. Clin. Nutr. 4: 609-618.

Hodson, A.Z. 1970. Conversion of ferric to ferrous iron in weight control dietaries. J. Agr. Food Chem. 18: 946-947.

Höglund, S., and P. Reizenstein. 1969. Studies in iron absorption. V. Effect of gastrointestinal factors on iron absorption. Blood 34: 496-504.

Jackel, S.S., and F. Belshaw. 1971. Encapsulated ferrous sulfate protects baking mixes, flour from rancid flavors. Food Process. (Chicago) 32(5): 28-29.

Johnston, F.A., R. Frenchman, and E.D. Boroughs. 1948. The absorption of iron from beef by women. J. Nutr. 35: 453-465.

Josephs, H.W. 1958. Absorption of iron as a problem in human physiology; a critical review. Blood 13: 1-54.

Layrisse, M., and C. Martinez-Torres. 1971. Food iron absorption: iron supplementation of food. Progr. Hematol. 7: 137-160.

Layrisse, M., and C. Martinez-Torres. 1972. Model for measuring dietary absorption of heme iron: test with a complete meal. Amer. J. Clin. Nutr. 25: 401-411.

Layrisse, M., C. Martinez-Torres, J.D. Cook, R. Walker, and C.A. Finch. 1973. Iron fortification of food: its measurement by the extrinsic tag method. Blood 41: 333-352.

Leichter, J., and M.A. Joslyn. 1967. The state of iron in flour, dough, and bread. Cereal Chem. 44: 346-352.

Leverton, R.M. 1941. Iron metabolism in human subjects on daily intakes of less than 5 milligrams. J. Nutr. 21: 617-631.

Marsh, A., H. Long, and E. Stierwalt. 1959. Comparative hematologic response to iron fortification of milk formula for infants. Pediatrics 24: 404-412.

McCay, C.M. 1953. Gustav B. von Bunge (January 19, 1844 - November 5, 1920). J. Nutr. 49: 3-19.

McCance, R.A., and E.M. Widdowson. 1937. Absorption and excretion of iron. Lancet 2: 680-684.

McCance, R.A., and E.M. Widdowson. 1938. The absorption and excretion of iron following oral and intravenous administration. J. Physiol. 94: 148-154.

McMillan, T.J., and F.A. Johnston. 1951. The absorption of iron from spinach by six young women, and the effect of beef upon the absorption. J. Nutr. 44: 383-398.

Moe, P.J. 1963. Iron requirements in infancy; longitudinal studies of iron requirements during the first year of life. ACTA Paediat. Suppl. 150: 67 pp.

Moore, C.V., and R. Dubach. 1956. Metabolism and requirements of iron in the human. J. Amer. Med. Ass. 162: 197-204.

Moore, C.V., W.R. Arrowsmith, J. Welch, and V. Minnich. 1939. Studies in iron transportation and metabolism. IV. Observations on the absorption of iron from the gastrointestinal tract. J. Clin. Invest. 18: 553-580

Moore, C.V., R. Dubach, V. Minnich, and H.K. Roberts. 1944. Absorption of ferrous and ferric radioactive iron by human subjects and by dogs. J. Clin. Invest. 23: 755-767.

Moore, C.V., V. Minnich, and R. Dubach. 1943. Absorption and therapeutic efficacy of iron phytate. J. Amer. Diet. Ass. 19: 841-844.

Morris, E.R., and F.E. Greene. 1972. Utilization of the iron of egg yolk for hemoglobin formation by the growing rat. J. Nutr. 102: 901-908.

Motzok, I., M.D. Pennell, and M.I. Davies. (1973). Factors affecting the biological availability of iron from food grade supplements. Proceedings, IX International Congress of Nutrition, Mexico City, Mexico, September 5, 1972. (In press)

Nakamura, F.I., and H.H. Mitchell. 1943. The utilization for hemoglobin regeneration of the iron in salts used in the enrichment of flour and bread. J. Nutr. 25: 39-48.

Niccum, W.L., R.L. Jackson, and G. Stearns. 1953. Use of ferric and ferrous iron in the prevention of hypochromic anemia in infants. Amer. J. Dis. Child. 86: 553-567.

Pirzio-Biroli, G., and C.A. Finch. 1960. Iron absorption. III. The influence of iron stores on iron absorption in the normal subject. J. Lab. Clin. Med. 55: 216-220.

Pirzio-Biroli, G., T.H. Bothwell, and C.A. Finch. 1958. Iron absorption. II. The absorption of radioiron administered with a standard meal in man. J. Lab. Clin. Med. 51: 37-48.

Pla, G.W., and J.C. Fritz. 1970. Availability of iron. J. Ass. Offic. Anal. Chem. 53: 791-800.

Pla, G.W., and J.C. Fritz. 1971. Collaborative study of the hemoglobin repletion test in chicks and rats for measuring availability of iron. J. Ass. Offic. Anal. Chem. 54: 13-17.

Ranhotra, G.S., F.N. Hepburn, and W.B. Bradley. 1971. Availability of iron in enriched bread. Cereal Chem. 48: 377-384.

Reimann, F., and F. Fritsch. 1930. Vergleichende untersuchungen zur therapeutischen wirksamkeit der eisenverbindungen bei den sekundären anämien. Z. Klin. Med. 115: 13-40.

Robscheit-Robbins, F.S., and G.H. Whipple. 1925. Blood regeneration in severe anemia. II. Favorable influence of liver, heart and skeletal muscle in diet. Amer. J. Physiol. 72: 408-418.

Saylor, L., and Finch, C.A. 1953. Determination of iron absorption using two isotopes of iron. Amer. J. Physiol. 172: 372-376.

Schulz, J., and N.J. Smith. 1958. A quantitative study of the absorption of food iron in infants and children. Amer. J. Dis. Child. 95: 109-119.

Sharpe, L.M., W.C. Peacock, R. Cooke, and R.S. Harris. 1950. The effect of phytate and other food factors on iron absorption. J. Nutr. 41: 433-446.

Stearns, G., and D. Stinger. 1937. Iron retention in infancy. J. Nutr. 13: 127-141.

Steinkamp, R., R. Dubach, and C.V. Moore. 1954. Absorption of radioiron from iron enriched bread. Fed. Proc. Fed. Amer. Soc. Exp. Biol. 13 (Part 1): 479. (Abstract 1572)

Steinkamp, R., R. Dubach, and C.V. Moore. 1955. Studies in iron transportation and metabolism. VIII. Absorption of radioiron from iron-enriched bread. Arch. Intern. Med. 95: 181-193.

Street, H.R. 1943. A study of the availability of the iron in enriched bread. J. Nutr. 26: 187-195.

Summerfeldt, P. 1935. Iron and its availability in foods. Trans. Sect. Pediatr. Amer. Med. Ass., 214-220.

Theuer, R.C., K.S. Kemmerer, W.H. Martin, B.L. Zoumas, and H.P. Sarett. Effect of processing on availability of iron salts in liquid infant formula products; experimental soy isolate formulas. J. Agr. Food Chem. 19: 555-558.

Theuer, R.C., W.H. Martin, J.F. Wallander, and H.P. Sarett. 1973. Effect of processing on availability of iron salts in liquid infant formula products. Experimental milk-based formulas. J. Agr. Food Chem. 21: 482-485.

Tisdall, F.F., T.G.H. Drake, and A. Brown. 1930. A new cereal mixture containing vitamins and mineral elements. Amer. J. Dis. Child. 40: 791-799.

Tobey, J.A., and W.H. Cathcart. 1941. Fortification and restoration in baking and dairy industries. Ind. Eng. Chem. 33: 714-716.

Turnbull, A., F. Cleton, and C.A. Finch. 1962. Iron absorption. IV. The absorption of hemoglobin iron. J. Clin. Invest. 41: 1897-1907.

Underwood, E.J. 1938. A comparison of ferrous and ferric iron in the nutrition of the rat. J. Nutr. 16: 299-308.

Vellar, O.D., C. Borchgrevink, and H. Natvig. 1968. Iron-fortified bread; absorption and utilization studies. Acta Med. Scand. 183: 251-256.

Waddell, J., and G.H. Kennedy. 1947. Pages 452-456 *in* W.J. Dann and G.W. Satterfield, eds. Estimation of the vitamins. Biological symposia, vol. 12, The Jacques Cattell Press, Lancaster, Pa.

Waddell, J., H. Steenbock, C.A. Elvehjem, and E.B. Hart. 1928. Iron in nutrition. V. The availability of the rat for studies in anemia. J. Biol. Chem. 77: 769-775.

Waddell, J., H.F. Sassoon, C.J. Carr, and K.D. Fisher. 1972a. Clinical research protocols to elucidate the possible hazards of increased iron enrichment of cereal products. Life Sciences Research Office, Federation of American Societies for Experimental Biology, 33 pp.

Waddell, J., H.F. Sassoon, K.D. Fisher, and C.J. Carr. 1972b. A review of the significance of dietary iron on iron storage phenomena. Life Sciences Research Office, Federation of American Societies for Experimental Biology, Bethesda, Md. PB-218 836; National Technical Information Service, Springfield, Va. 81 pp.

Walsh, R.J., I. Kaldor, I. Brading, and E.P. George. 1955. The availability of iron in meat: Some experiments with radioactive iron. Australas. Ann. Med. 4: 272-276.

Weintraub, L.R., M.E. Conrad, and W.H. Crosby. 1965. Absorption of hemoglobin iron by the rat. Proc. Soc. Exp. Biol. Med. 120: 840-843.

Weintraub, L.R., M.B. Weinstein, H.J. Huser, and S. Rafal. 1968. Absorption of hemoglobin iron: The role of a heme-splitting substance in the intestinal mucosa. J. Clin. Invest. 47: 531-539.

Whipple, G.H., and F.S. Robscheit-Robbins. 1925. Blood regeneration in severe anemia. I. Standard basal ration bread and experimental methods. Amer. J. Physiol. 72: 395-407.

Whipple, G.H., and F.S. Robscheit-Robbins. 1930. Blood regeneration in severe anemia. XVI. Optimun iron therapy and salt effect. Amer. J. Physiol. 92: 362-377.

Witts, L.J. 1936. The therapeutic action of iron. Lancet 1: 1-5.

IX. LIST OF CONSULTANTS

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THE BIOAVAILABILITY OF IRON SOURCES AND THEIR

UTILIZATION IN FOOD ENRICHMENT

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APPENDIX A

Considerations of the Effect of Physical Properties on the Bioavailability of Elemental Iron Powders

John Patrick, Jr. *

It is well recognized that many of the data recorded through the years on the use of elemental iron powder (usually referred to as reduced iron or ferrum reductum) for food enrichment lacked essential chemical, and more importantly, physical identification. This has provided a poor historical base from which modern scientists could advance systematically. More recently, investigators have been puzzled by erratic behavior of "similar reduced irons" used in their bioavailability experiments. The fact is that there are several members of the elemental iron powder family, each one differing from the other because of its method of manufacture, granulation and classification. Most producers of food grade iron powder are in the business of making all sorts of metal powders and to them the need for chemical and physical identification is foremost. The intention here is to describe those characteristics of iron powders, from a metallurgical view, which may be meaningful to the food industry, as well as identifying those types of iron powders known to be produced for this purpose.

Use of reduced iron as a descriptive term for all food grade iron powders is avoided here because it is a metallurgical term for just one member of the iron powder family.

Reduced iron is made by reduction of ground iron oxide with hydrogen or carbon monoxide at an elevated temperature. The purity of the product is dictated by the purity of the iron oxide used. Most domestic reduced irons are made from mill scale rather than iron ore because the former is more pure. However, these products have the lowest purity of the food grade iron powders used. The basic impurities include C, Mg, Al, Si, P, S, Cr, Mn, Ni, and Cu, many of which are present as oxides and appear in the "acid insolubles" analysis. Because of the nature of the process, iron mill scales reduced in carbon monoxide tend to have higher carbon (graphitic and/or combined) and sulfur. By far, the greatest impurity in any iron powder is oxygen, and most of this

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* Glidden-Durkee Division of SCM Corporation, Metals Group, Johnstown, Pennsylvania.

occurs as a thin film, surface oxide (ferrosic, also called ferrosoferric) as the produce is mechanically ground to powder (more surface area is formed and exposed to atmosphere). There is reason to believe that surface oxide is essential to improve bioavailability of iron powder. Its presence defines an "active" surface which is more prone to additional attack by stomach acids. In the case of reduced iron, however, there is in addition the probability that some of the original iron oxide (ferric) will remain as a core in the center of the particles and this portion will contribute little or no relative biological value.

Reduced iron powder is a brittle material which lends itself well to comminution by ball milling, hammer milling, or attrition milling. The particle shape is considered sponge-like, irregular and porous (Figure A-1). The particle consists of a number of small, equiaxed grains, along whose boundaries can be found those impurity inclusions which are not alloyed with iron. These together with iron oxide inclusions provide weak-points which contribute to its friability.

Electrolytic iron powder is produced by electrolytic deposition of a hard, brittle metal which is mechanically comminuted. The iron is produced domestically using chemically pure iron anodes, a ferrous sulfate bath (electrolyte), and thin stainless steel cathode sheets onto which the iron migrates. These sheets are removed from the bath after a standard plating cycle, washed to remove soluble salts, dried, then flexed to remove the brittle deposit. These "fragments" are then mechanically ground to a finely divided powder. Insoluble contaminants originating from the anode fall to the bottom of the bath as a sludge, while electrochemical conditions are set to favor the migration primarily of iron ions to the cathode. Impurities which remain are usually at levels of hundreds of parts per million or less. Again, as was described above, surface oxide is the major "impurity".

The particle shape of electrolytic iron powder is described as irregular, dendritic or fernlike (Figure A-2) from which it receives its high surface factor. Unlike reduced iron, the grains in electrolytic particles are less symmetrical. Powders obtained by the electrolytic method are generally somewhat harder than those produced by reduction so that grinding to powders with greater content of subsieve size is possible.

The extent to which carbonyl iron powder is produced in the United States for food enrichment purposes has not been established. Its production and use abroad has been reported. The unusual property of

extremely fine particle size presupposes its use in this country and, therefore, warrants mention here. The method of manufacture involves the treatment of scrap iron or reduced iron with carbon monoxide under heat and pressure. The resulting iron pentacarbonyl, $Fe(CO)_5$, is later decomposed under controlled conditions, yielding an iron powder and carbon monoxide gas. At this point the major impurity is carbon (about 1%) and a further reduction in wet hydrogen is necessary to remove most of it. The powder has particles ranging in size from 0.5 to 10 microns in diameter and is of high purity.

The carbonyl iron particle is very nearly spherical in shape (Figure A-3) and often as a cluster of several spheres "fused" together. The structure of the particle is characterized by concentric shells arranged in onion-skin fashion. The particle is very dense, smooth and hard-skinned, not readily prone to surface oxidation (oxygen content is about 1/5 that of reduced iron or electrolytic iron.) The major impurities are oxygen, carbon and nitrogen, while lesser amounts of Si, Cr, Mn and Ni are usually present at about the same level as in electrolytic iron. The carbonyl process is the most costly of those discussed here.

This completes the introduction of the members of the elemental iron powder family known to be used in food enrichment:

1. Reduced Iron
   - By Hydrogen
   - By Carbon Monoxide
2. Electrolytic Iron
3. Carbonyl Iron

They are produced differently, each exhibits its own special set of properties, and, unfortunately, past practice has been such that each has been identified in the literature as ferrum reductum or reduced iron.

A powder is generally considered to consist of discreet particles of dry material with a maximum dimension of 1 mm. While the use of coarse iron powder in the food industry is known to exist, most usage has been restricted to -100 mesh (149 microns or smaller) powder as presently specified under Iron, Reduced in the Food Chemicals Codex, Second Edition, 1972. In recent months there has been much discussion regarding the use of finer iron powder for food enrichment because increased solubility (and bioavailability) is associated with a more finely divided iron. With a given member of the iron powder family this is essentially true, but the theory does not exactly translate from member

to member. It is appropriate now to discuss those properties which relate to an iron powder's particle size and shape, and which will permit an investigator to suitably identify his sample. As has been shown, there are different shapes among the forms of iron powders discussed, and several methods for characterization may have to be used to get a comprehensive analysis of a given powder. In any discussion of particle physics the ideal model would be a true sphere, and the greater the departure from this shape, the more difficult it is to apply fundamental laws and predict property measurements. It should be mentioned at this point that new techniques for property measurement are constantly being developed, but it is far beyond the scope of this paper to account for even a portion of them. Instead, those basic measurements used by the powder metallurgy industry will be discussed.

Sieving is the best known and most widely used method of size analysis because it is simple, fast and relatively inexpensive. The method is described fully by ASTM Standard B 214. Both laboratory and production sieves are available covering a range of apertures from 0.3 to 0.0017 inch. In terms of U.S. sieves of most importance to the present discussion, it may be noted that the 100 mesh sieve has an aperture of 0.0059 inch; the 325 mesh sieve an aperture of 0.0017 inch. In making a comparison between different iron powders it is relatively easy to record the percent of each which remains on top of the 100, the 200, and the 325 mesh, and that which passes through the 325 mesh sieve. Sieve "blinding" will occur when particles have partly passed through the mesh and are held by their maximum diameter in the aperture. Analytical reproducibility will be poor unless the sieves are periodically cleaned (ultrasonic cleaning appears to work best).

Measurement of particle sizes below 325 mesh (44 microns) will require what is known as "subsieve" methods. Probably the most common method involves the Fisher Subsieve Sizer (Fisher Scientific Co., Pittsburgh, Pa.). The instrument was developed in the 1940's and has gained widespread use in the metal powder industry. Pressure loss of air caused by friction with particle surfaces is measured, with the packing of small particles offering more resistance to air flow than one of large particles. The test is quick and the value (not absolute) obtained is called a "Fisher Number" which is reported as an average particle size in microns. The method is best suited for comparing different lots of the same commercial powder, and is described by ASTM Standard B 330.

Another subsieve measuring apparatus found in many powder metallurgy laboratories is the Roller Air Analyzer (American Instrument

Co., Silver Spring, Md.). This is an air elutriation method which is accurate for solid spheres and which becomes less accurate with departure from that shape. This separator works on the basis of Stokes' law as applied to the fall of spherical particles in an upward uniform flow of air. A selected range of small sized particles (say 0-10 microns) is carried away by an applied air stream and collected in a thimble. The apparatus is then adjusted and the next larger particle size fraction (10-20 microns) is separated. This is repeated for the 20-30 and 30-40 micron fractions, and what remains behind in the original sample tube is the 40-44 micron "oversize." On a given lot of iron powder, only the minus 325 mesh portion can be analyzed by this method, so that a particle size analysis for a 100 mesh powder would be presented by showing a sieve analysis and also a Roller analysis (indicating the percentage distribution in the "subsieve" portion of that particular lot). Where a Fisher Number determination requires only a few minutes to complete, a fractionation by the Roller method can take as long as eight hours; however, the data are obviously more meaningful. This elutriation method is covered by ASTM Standard B 293.

The property of particle shape has special importance since it effects surface area, apparent (bulk) density, permeability (as in the Fisher No. test) and flow characteristics of the powder. It has been found that the best characterization of shape will be an approximation, and for the necessity of simplicity and better interpretation it is best treated as a two dimensional model rather than three. This can be justified for most powders since from the major axis (the longest dimension), usually two axes have practically the same dimensions. The microscope is the most dependable tool for examination of particle shape, and photomicrographs can be made for the purpose of comparing lot with lot, or member with member in the iron powders. The value of estimating particle shape of different samples and relating this to bioavailability provides still another means for identifying experimental behavior.

Another property of interest is the apparent density of a powder population. This should not be confused with the true density or specific gravity of the material composing the particles (eg. 7.86 g/cc for iron). Apparent density is defined as the weight of a mass of powder loosely heaped in a given volume and is expressed in grams per cubic centimeter. Most commercial metal powders used are minus 100 mesh and have a "natural" particle size distribution which means that in a loose packing mode, the voids between near-sized particles are filled with smaller particles with the net result of minimal porosity in the bed

and a high apparent density (eg. 2.50 g/cc). As the particle size distribution is narrowed, say a minus 325 mesh powder is now to be tested, there are no larger particles to create accommodations for the ultra fines (which represent tremendous surface area). Now large voids are formed by arching or bridging of adjacent particles and the result is a low apparent density (eg. 2.00 g/cc). With irregular shaped particles like the reduced irons and electrolytic iron, bridging is more pronounced in the finer granulations than it would be with carbonyl iron. There are at least a dozen other factors which influence apparent density, but the intent here is to draw attention to another standardized, convenient and reproducible test which is helpful in identifying iron powders. ASTM standards are provided for free-flowing metal powders (B 212) and non-free-flowing metal powders (B 417) which usually are the finer powders. The flowmeter funnel, density cup, and stand are available as a unit from Alcan Metal Powders, Inc., Box 290, Elizabeth, New Jersey, 07207.

An effort has been made here to acquaint the scientist or the user of food grade iron powders with basic methods for identifying the sample of the purchased product, and beyond that, to make them aware of the differences among commercially available powders so that proper identification will be demanded. As an example, a recent paper on food iron spoke of the use of ferrum reductum in Sweden with 95% below five micron particle size. Most would agree that this would exhibit super-bioavailability; powder metallurgists might expect pure iron so finely divided to be pyrophoric, burning to incandescence when the cover is removed from the container. Samples of this powder have been tested in this country during the past two years showing a puzzling, less than super-bioavailability. The material has just been examined microscopically and is shown in the scanning electron micrograph in this paper as Figure 3. The particles are sufficiently distinctive in appearance and character as to lend further support to the contention that it is wrong to include all iron powders under the one name, reduced iron or ferrum reductum.

At many roundtable meetings the purity of food grade iron powder and its relation to solubility and bioavailability is often a subject for discussion. "Iron powder produced today is too pure." Which form of iron powder? If one is referring to electrolytic or carbonyl iron then this statement is not true. Standard manufacturing processes for these two remain essentially the same and they involve complete breakdown of the starting material and a "rebirth" of metallic iron. If one is referring to either of the reduced irons the statement

is still not true. Here also the process remains essentially the same. However, the purity of the product is dictated by the purity of the mill scale raw material (in a solid to solid state reaction such as this), and this purity can be expected to go down. The reason for this is the ever increasing build-up of impurities in scrap iron, a serious problem in steel mills today. The increasing cost of mining ore and the vanishing deposits do not warrant optimism. So then, it appears that today's iron being too pure is a weak statement, and possibly the investigator was unwittingly comparing apples to oranges. As mentioned earlier, the impurity oxygen, present as a thin film surrounding the particle, may be essential in triggering dissolution.

Finally, it should be mentioned that producers of food grade elemental iron powders are equipped to measure the properties discussed and they report most of them in their lot analysis. It is important that the user take advantage of the data and other services offered by the manufacturer.

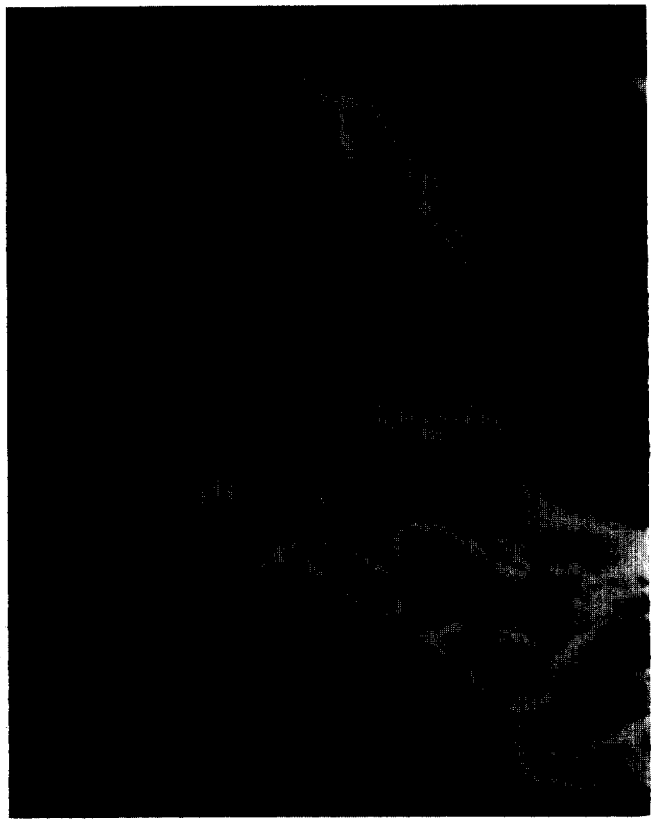


Figure A-1
Reduced iron particle,
5000X, Glidden B-131

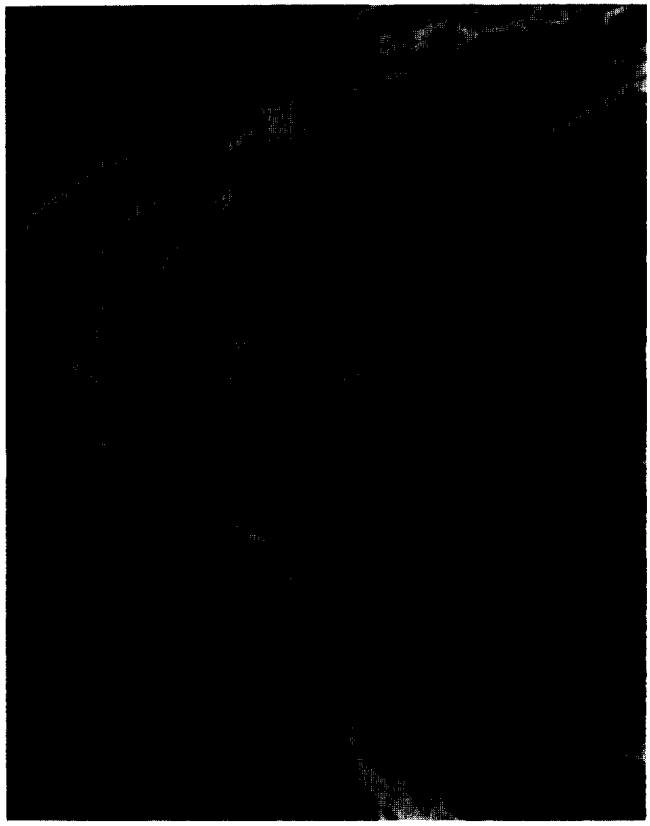
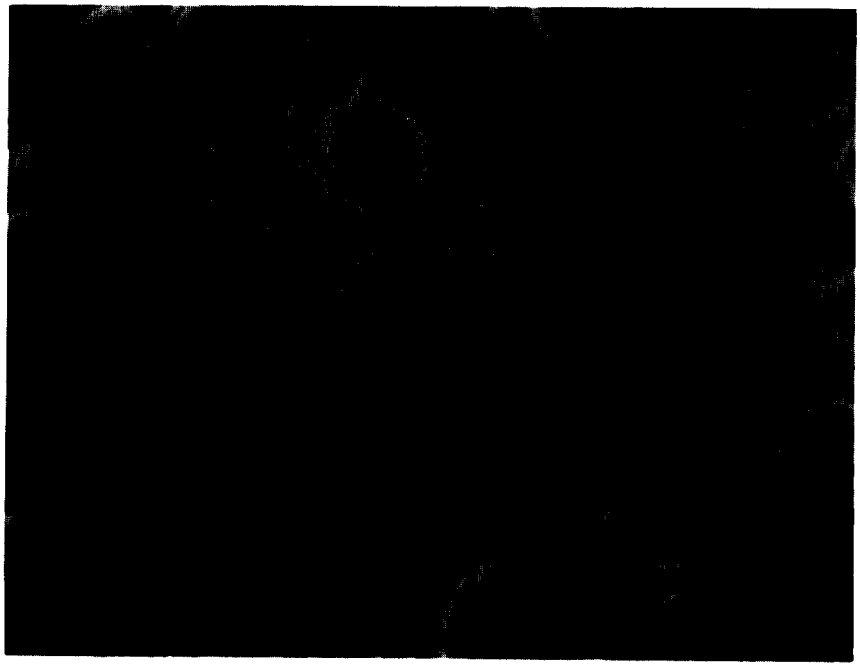


Figure A-2
Electrolytic iron particle,
5000X, Glidden B-131

Figure A-3
Carbonyl iron powder, 5000X.



Sample received from Sweden: stated to be typical of "ferrum reductum" used for flour enrichment.

APPENDIX B

Comments on the Development of an Official Assay for Determining the Relative Bioavailability of Iron Sources

James Waddell

Assay Design

The hemoglobin repletion method has been used for more than 40 years in one way or another to assess the absorption and utilization of iron from different sources. The method would appear to possess the characteristics required of a quantitative bioassay procedure: it is possible by dietary means to produce a relative iron deficiency in test animals; there is a dose-related response to iron additions to the diet; and these responses may be measured with accuracy and relative ease. It would seen strange if, among the many procedures that have been described over the years for the assay of biological products, one were not found that would be applicable to the determination of the relative biological value of iron sources. It is of interest, therefore, to note that Blumberg and Arnold (1947), in assessing with iron-deficient rats the relative biological value of ferric orthophosphate, used the assay design and the numerical procedure for estimating relative potency and its standard error described by Waddell and Kennedy (1947) for the assay of vitamin D with chicks. The results of Blumberg and Arnold showed the iron of ferric orthophosphate, in comparison with that of exsiccated ferrous sulfate, to have a value of 25.2 ± 2.0 percent, indicating a very high degree of precision for the assay as carried out by them.

This assay procedure is a well-known method for assessing the biological efficacy of "unknowns" in terms of a reference standard where the response bears a linear relationship to the logarithm of the dose. This relationship has been fully established for many of the biological assays, and applied except where the responses fall near the extremes of the dose-response curve. Multiple graded doses of both standard and unknown(s) must be given so that a straight line response for each may be calculated by the method of least squares. The assumption is made that the unknown sample does not produce a qualitatively different response from that of the standard; that the difference is only quantitative. Hence, it is assumed that the slopes of the response of the standard and the unknown are the same and they are made parallel by computing a single combined slobe (b_C). The relation of any unknown to the standard may be visualized by the horizontal distance between the two response lines which represents, of course, the difference on the log dose axis between

the amounts of the two compounds required to produce the same response. The final step in computing the relative value of the unknown is to transform this difference into original units through the use of its antilogarithm.

The above assay procedure also permits an estimate of the reliability or precision of the determination to be calculated. Using the differences between the observed responses and the computed lines, the number of groups of animals and the ratio of these standard deviations to the combined slope of the assay, a standard error of the potency may be computed.

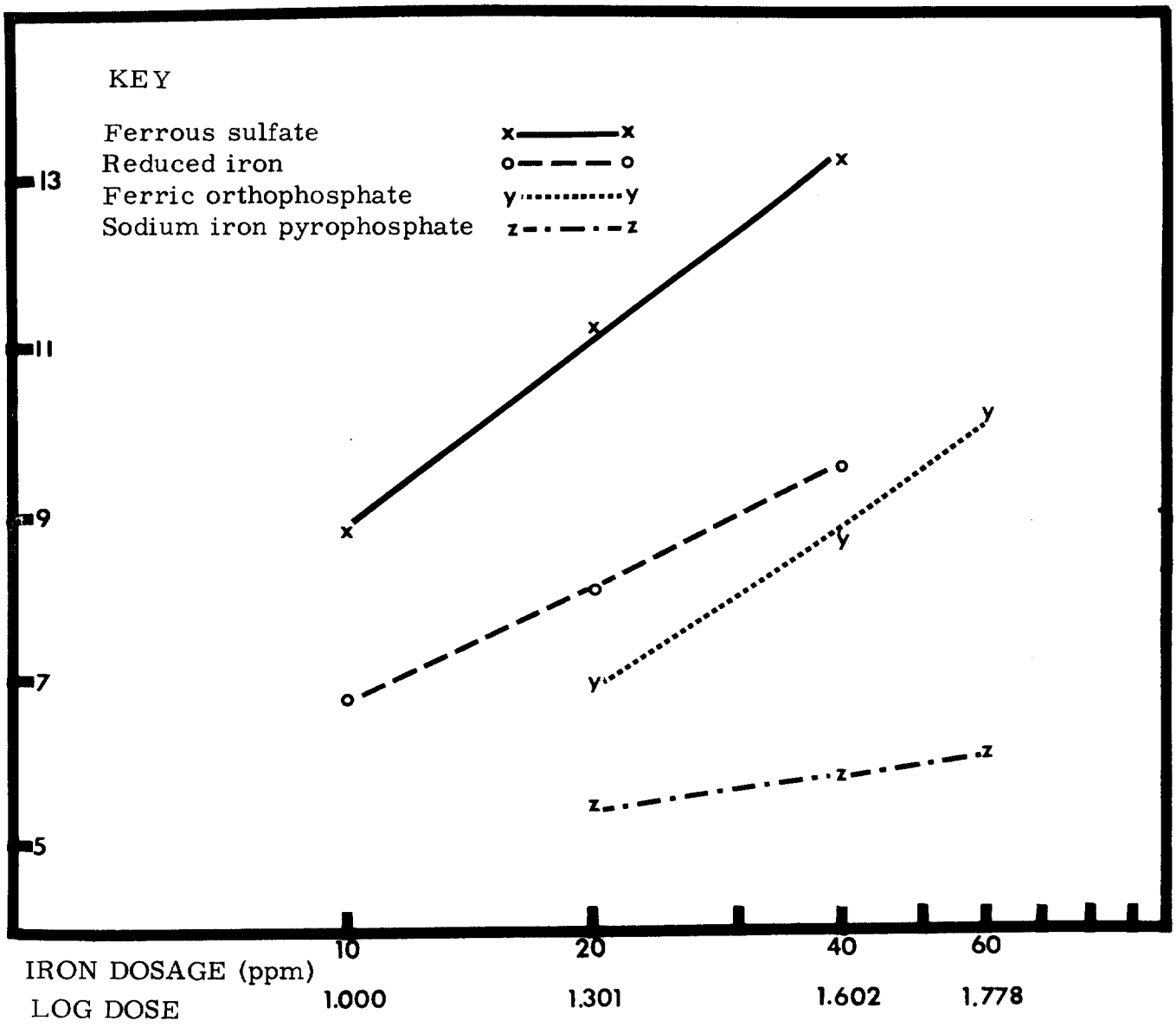
Amine *et al.* (1972) have applied the slope-ratio method of assay as a means of estimating the relative biological availability of the iron in various sources, including the four most used in the enrichment of foods. The distinguishing feature of this method is the assumption that the response is a linear function of the dose rather than of the log dose, a relationship which has been found to apply in many microbiological assays. The dosage levels are measured from zero on an arithmetic scale and the regression of response with increasing dose of each sample is fitted by a straight line, the individual lines converging at zero dose. Because the potency of an unknown, relative to the standard, can be determined from the ratio of the slopes of their dose-response lines, such assays are referred to as slope-ratio assays.

It is obvious that the physiological response can not be a linear function of both dose and log dose; at least not over any great range of doses. Therefore, in setting about to develop an "official" assay method for iron compounds a choice must be made between the two procedures outlined above. Evidence of departure from linearity in responses to a relatively short range of doses was noted by Amine *et al.* (1972) in the four assays which they carried out by the slope-ratio procedure, and the responses of several groups receiving the highest doses of the more effective supplements were excluded from the computation of relative potency. In Figure B-1 the hemoglobin responses (g/100 ml blood) exhibited by four of the iron sources studied in Experiment 1 of Amine *et al.* (1972) have been plotted against the logarithms of the doses for the purpose of examining the linearity of the responses. Amine *et al.* felt compelled to exclude from their estimates of potency the responses to the 40 ppm doses of both ferrous sulfate and reduced iron in that experiment. As shown in Figure B-1 there is no reason to believe that the exclusion of these data would be permissible in a log dose parallel lines assay. Indeed, in each of the four supplements the regression of response on log dose appears to be acceptably linear.

On the basis of the above limited comparison, together with the excellent linear regression of hemoglobin level on log dose demonstrated by Blumberg and Arnold (1947), it would seem that the log dose parallel

FIGURE B-1

HEMOGLOBIN RESPONSE IN RELATION TO LOGARITHM OF DOSE



Prepared from the figures in Experiment 1, Amine *et al.* (1972). Lines drawn by inspection only.

lines method of assay would be the better choice for an official method for determining the relative bioavailability of the iron in different sources.

In the light of the above brief discussion of two well-established assay procedures, it will be clear that a test involving single levels of standard and unknowns will provide very limited information as to relative efficacy. There is no assurance that the responses fall on linear portions of the curves and there is limited opportunity to assess inherent variability. It is a minimal improvement to provide multiple graded doses of only the standard, except in preliminary assays to obtain some idea of the relative potency of unknowns. To obtain a precise comparison, at least to provide a means for estimating precision, multiple doses of both standard and unknowns must be used. Three levels would appear to be a minimal requirement; more than three may be advantageous. In log dose assays the doses chosen should preferably be in geometric progression (equal log interval between succeeding doses). A common progression is a doubling of one dose to provide the next (5, 10, 20, 40, etc.; interval = log 2) but others may be used.

Another important consideration in the selection of doses is that the responses produced by unknowns should be in a range comparable with those of the standard. An illustration of what would appear to be inappropriate dosage may be seen in Figure B-1 in the case of sodium iron pyrophosphate. The responses to the doses fed are so meagre as to suggest that they may lie on the extreme lower portion of the dose-response curve where the responses may not be linear. Certainly, much higher doses should be fed in a subsequent assay. Blumberg and Arnold (1947), in comparing ferric orthophosphate with ferrous sulfate, used four doses of each material; the doses of standard ranged from 5.25 to 42 ppm separated by a log interval of 2, while the doses of the orthophosphate ranged from 8.4 to 131.2 ppm using a log interval of 2.5. Excellent linear and fully comparable regressions were obtained.

Animal Species

Much of the recent investigation of the comparative bioavailability of iron sources has involved the use of both weanling rats and baby chicks. Both species appear to be satisfactory test animals but there are suggestions of some variation between them in response to certain iron sources. Because of this no suitable test method could countenance the use of both species as official. In the interest of expediting the development of a suitable method it would seem wise to use the rat rather than the chick.

Measurement of Response

Similarly, in recent investigations, authors have tended to report response to iron supplementation in terms of both hemoglobin level and packed cell volume (hematocrit). Both measurements appear to supply the same information but, on physiological grounds, hemoglobin production would seem to be a more direct and specific measure of iron availability in iron-deficient animals and this should be made part of any official method. The reporting of hematocrit values, where they add nothing to the interpretation, should be discouraged.

Curative Versus Prophylactic Assay

Historically, the hemoglobin repletion method, as its name implies, made use of rats which had been rendered distinctly iron-deficient before they were given supplements containing iron. The basic comparison under these conditions may be thought of as the difference between the supplements in the degree of "cure" of iron deficiency which each brought about. More recently, assays have been carried out in which different levels of iron supplements were added to the iron-low diet of normal young animals at the start of the experiment (Amine *et al.*, 1972; Motzok *et al.*, 1972). Here the basic comparison between the supplements would be assessed by the degree to which iron deficiency was prevented.

The only advantage of the prophylactic assay is the saving of time, effort, and materials. These are important considerations if there is no sacrifice of precision in the results of the assay as compared with the curative. The findings of the above authors do no provide an answer. Theoretically, the prophylactic procedure would seem to be somewhat inferior because the animals, starting with a normal hemoglobin level, are likely to use a lesser percentage of the absorbed iron in the synthesis of hemoglobin during a short test period. Because hemoglobin level is the measure of response it is possible that the prophylactic assay could be less discriminating than the curative in the comparison of iron sources. One would hope that, at this stage in the development of an official assay procedure, accuracy and precision would take priority over considerations of a shorter and less expensive test. Later, if experience shows that the prophylactic assay is fully adequate, changes in the method could be introduced.

Numerical Procedure in Computing Relative Potency and its Standard Error

One of the important characteristics of a well-designed bioassay is that it provides for an objective numerical method for calculating relative potency and its standard error. There are well-established

using the data derived from the assay by which the individual statistics may be computed. These equations, applying to the log dose parallel lines method, are to be found in standard treatises, such as that of Bliss and White (1967). The contribution of Waddell and Kennedy (1947) was to illustrate the step-by-step computations based on bone ash responses to increasing doses of vitamin D; Blumberg and Arnold (1947) demonstrated that the same methods of computation applied to hemoglobin responses to increasing doses of iron.

Such a numerical procedure for computing potency and error should be made part of any official method for determining the bioavailability of the iron in different sources.

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